Universidade de Aveiro Departamento de Biologia
2007

Isabel Maria de Carvalho Benta Santos Oliveira

Spatial and temporal evolution of imposex in *Nucella lapillus* (L.) populations from North Wales

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Sr. Prof. Doutor Carlos Miguel Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Sr. Prof. Doutor Fernando José Mendes Gonçalves
Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

Sr. Doutor Miguel Alberto Fernandes Machado Santos
Investigador Auxiliar do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Sr. Prof. Doutor Carlos Miguel Miguez Barroso
Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Quero agradecer ao meu orientador, Sr. Prof. Doutor Carlos Miguel Miguez Barroso, todo o acompanhamento interessado destes últimos quatro anos. Agradeço, particularmente, a forma carinhosa e paciente como me acompanhou na escrita desta tese e como, através do seu exemplo e entusiasmo, tanto me ensinou, fazendo com que todos os dias fossem uma descoberta constante do lado mais interessante, mas também mais responsável, da ciência. Muito obrigada por, ao longo de todo este tempo, ter sido um exelente professor e também um amigo.

Agradeço ao Sr. Prof. Doutor Chris Richardson a forma como se disponibilizou para me receber na Escola de Ciências Marinhas, em Menai Bridge (UK). Obrigada pela sua incansável e total disponibilidade que nos levou a percorrer a costa do País de Gales, em tão pouco tempo, e pela alegria contagiante com que foi realizado este trabalho.

Agradeço à Dr.ª Ana Sousa a amizade e a celeridade com que foi acedendo prontamente aos meus pedidos. Muito obrigada pelas semanas de intenso esforço, para que a realização das análises químicas das amostras, relativas a este trabalho, fossem executadas. Obrigada também pelas discussões científicas.

Um agradecimento especial à Dr.ª Susana Galante Oliveira por toda a paciência, pelas saídas de campo, pelas conversas, pelas viagens... por todo o acompanhamento que me deu e ainda continua a dar! Mais do que uma companheira de trabalho, é uma grande amiga.

Agradeço à Luciana e à Margarida a preciosa ajuda não só nas amostragens em 2004/2005 mas também na adaptação a um novo País. Foi bem mais fácil com a vossa ajuda. Obrigada também à Raquel por todo o cuidado e preocupação com o envio das amostras biológicas para Portugal. Agradeço a toda a equipa do LEME – aos que ainda por cá estão e àqueles que já saíram; todos contribuíram, para o clima de amizade, alegria e entre-ajuda no laboratório. É bom trabalhar assim!

A todos os meus amigos: Ana, Nuno, Inês, Silvia, João; obrigada por, ao longo deste tempo, não me deixarem desanimar.

Quero também agradecer à minha família: foram, são e sempre serão o meu porto seguro. Não me consigo imaginar sem todo o vosso apoio, carinho, amor e paciência. Guardo comigo todos os valores e ensinamentos que sempre me incutiram.

Por último, quero agradecer ao Denis pelo apoio incondicional, porque mesmo longe consegue fazer com que continue a sentir a sua presença muito perto. Obrigada por seres sempre o meu melhor amigo, por me fazeres perceber que, mesmo nas horas mais complicadas, estamos os dois juntos, sem restrições.

resumo

O impossexo - superimposição de caracteres masculinos em fêmeas de prosobrânquios - é uma resposta biológica altamente específica à exposição de compostos organoestânicos e tem sido utilizado para monitorizar os níveis de poluição por TBT e TPT, nas costas Atlânticas.

A relação causa-efeito entre o TBT e o impossexo foi já comprovada por vários autores. No entanto, estudos recentes efectuados na Ria de Aveiro sugeriram que duas populações poderiam estar a desenvolver diferentes sensibilidades, na resposta do impossexo, à contaminação por TBT. Por forma a clarificar esta hipótese, um grupo de animais de cada local foi exposto, em laboratório, a uma concentração nominal de 100ng TBT-Sn/L durante 45 dias. Verificou-se o desenvolvimento de impossexo em ambos os grupos de animais mas não foram observadas diferenças significativas entre ambas as respostas. Estes resultados suportam a ideia de que existe uma uniformidade geográfica na relação entre a intensidade do impossexo e os níveis de poluição por TBT.

Foi investigada a distribuição espacial do imposexo no Norte do País de Gales, através da observação de individuos da espécie *Nucella lapillus*, amostrados entre Novembro e Dezembro de 2006. Foi também efectuada a análise química aos organoestânicos presentes nos tecidos das fêmeas de *N. lapillus*. Os níveis de imposexo obtidos no presente trabalho foram comparados com dados de estudos semelhantes, efectuados na mesma zona desde 1987, de modo a caracterizar a evolução temporal da poluição por TBT nas últimas duas décadas. Foi possível observar um decréscimo global dos níveis de impossexo, o que denota a eficácia das medidas que restringem o uso de TBT no Reino Unido.

abstract

Imposex - superimposition of male characters onto prosobranch females - in the dogwhelk *Nucella lapillus* is a highly specific biological response to organotin exposure and has been used for monitoring TBT and TPT pollution throughout Atlantic coasts.

A cause-effect relationship between TBT and imposex was already reported by many authors. However, recent field studies have suggested the hypothesis that there could be different sensitivities in the imposex response of dogwhelks from two different sites in Ria de Aveiro. In order to clarify this hypothesis, dogwhelks from both sites were exposed, at the laboratory, to a nominal concentration of 100ng TBT-Sn/L for 45 days. TBT caused the development of imposex in both groups of animals but no significant different responses were observed between them. These results support the concept of a geographical uniformity of dogwhelks' imposex response to TBT pollution.

Spatial and temporal evolution of imposex around North Wales was investigated through the assessment of *N. lapillus* imposex, between November and December of 2006 along North Wales' coast (with respective organotin analysis of the dogwhelk female tissues) by the comparison of these data with data reported in similar previous studies of other authors. The effectiveness of the TBT restrictive measures in the UK were corroborated by the decreasing imposex values from 1987 to 2006.

Index

List of Figures

<u>Chapter 1</u>

<u>Chapter 2</u>

Chapter 3

List of Tables

List of Acronyms

AF – Antifouling

DMSO – Dimethylsulphoxide

%I – percentage of affected females

Imposex – super**impo**sition of male **sex**ual characters in females of gonochoric gastropods

FPL – Female Penis Length

FPLI – Female Penis Length Index

MPL – Male Penis Length

MPLI – Male Penis Length Index

OTs – Organotin compounds

RPS – Relative Penis Size

RPSI – Relative Penis Size Index

SD – Standard deviation

SE – Standard error

TBT – Tributyltin

TPT – Triphenyltin

U-test – Mann-Whitney U test

VDS – vas deferens sequence

VDSI – vas deferens sequence index

W-test – Wilcoxon matched pairs test

Chapter 1 – Introduction

1.1 – The historical use of organotin compounds

Organotin compounds (OTs) have been used for a wide range of applications including PVC stabilizers, antifoulants, agrochemicals pharmaceuticals, wood and stone preservatives, fungicides, catalysts and disinfectants (Bennett, 1996; Oehlmann *et al.*, 1998; Appel, 2004; Ohji *et al.*, 2007). These compounds are effective in very low concentrations, which make them advantageous in terms of formulation and costs. The maximal biological activity shown by organotins relies on the triorganotin form (Omae, 2003). The main example of that is tributyltin (TBT) and triphenyltin (TPT).

Organotin compounds were first developed as moth-proofing agents in the 1920s, and only later, in 1950, a group working under the supervision of G.J.M. van der Kerk discovered that organotins had many bactericidal and fungicidal applications (Omae, 2003). Tributyltin compounds (TBT oxide and TBT fluoride) were first used as molluscids to eradicate snail carriers of the human disease schistosomia (Power and Keegan, 2001). As a result of their effectiveness, it led to the addition of triorganotin compounds to the formulation of marine antifouling (AF) paints as biocide agents (Yebra *et al.*, 2004). The use of TBT in marine AF paints dates from the early 1960s.

Marine biofouling can be described as the undesirable accumulation of microorganisms, plants and animals on artificial surfaces immersed in seawater (Figure 1) (Yebra *et al.*, 2004). Fouling produces roughness on the bottom surfaces of the ship hull, increasing turbulence and drag. This results in a substantial increase, not only of the localized corrosion, fuel consumption and emissions (atmospheric and marine) but also on frequency of dry-docking inspection and maintenance of the vessels hull and, consequently, the costs (Axiak, 2003; Yebra *et al.*, 2004). The spread of non-indigenous species can also be an ecological problem (Howel and Behrends, 2006).

Figure 1 – Hulls of two heavily fouled ships (Yebra *et al.*, 2004).

As a consequence, AF paints were widely applied in boats and also in aquaculture cages to discourage the growth of marine organisms. The main organotins applied in these paints were TBT and, less frequently, TPT (Alzieu *et al.*, 1988; Bennett, 1996).

Antifouling paints can be categorized into two groups depending on the way that the biocide is released. In the first group, the biocide is held in free association with the paint matrix and released exponentially over time. These paints are predominantly based on copper compounds and can be efficient for 50 to 80 weeks (Champ, 2000; de Mora, 1996). The self-polishing copolymer paints belong to the second group (TBT – SPC paints), which have been the most successful in combating biofouling on ships (Yebra *et al.*, 2004). The TBT molecule is chemically attached to a polymer backbone which is hydrostatically unstable. The biocide is released by chemical hydrolysis in a reaction that occurs at a constantly renewed surface layer (Champ, 2000; Yebra *et al.*, 2004). Aside from the high biocide release during the initial period, there is a constant leaching of it over time (Omae, 2003). This coating remains effective for 5 – 7 years (Champ, 2000; Yebra *et al.*, 2006) even under static conditions and the surface of the ship hull remains as smooth as when it was first painted (Proud, 1994; Jelic-Mrcelic *et al.*, 2006).

The effective life of TBT self-polishing co-polymers is more than the double that of other types of antifouling and hulls can be repainted without the need to remove any remaining old co-polymer coating (Omae, 2003). That is why it represents a considerable financial saving. On the other hand, the TBT is a potent biocide and is thought to be one of the most toxic substances ever deliberately introduced into natural waters by mankind (Goldberg, 1986).

1.2 – Distribution and fate of organotins

The degradation on OTs occurs naturally in the environment through a certain number of pathways such as biological cleavage and U.V. radiation (Omae, 2003). Nevertheless, abiotic degradation is very slow and, because of that, biodegradation is the most important route (Dowson *et al.*, 1996).

Degradation of OTs can be defined as the progressive removal of the organic group attached to the tin atom (Dowson *et al.*, 1996; Gadd, 2000; Omae, 2003).

$$R_4Sn \rightarrow \mathbf{R_3SnX} \rightarrow R_2SnX_2 \rightarrow RSnX_3 \rightarrow SnX_4$$

R represents a carbon (banded organic group) and X represents an inorganic substitute. The inorganic group as little or no effect on the biological activity of the compound, opposing to the organic group that origin the decline in the biological activity of the triorganotin form when is lost (Omae, 2003). R_4Sn compounds have low toxicity (Gadd, 2000) but the triorganotin form, which is represented in bold letter, is the most effective one. In general, TBT and TPT are the most toxic compounds within this group.

The large molecular weight and the lipid solubility of these trisubstituted tins are related with the theory that triorganotins exert toxic effects through interactions with membrane lipids (Gadd, 2000). Tributyltin has been also recorded as an effective compound in the inhibition of microbial processes and main interactions occurring at cellular membranes of chloroplasts or at the mitochondria in eukaryotes (Gadd, 2000).

Initially, TBT was thought to be an environmental friendly alternative to non-degradable and toxic heavy metals such as copper, mercury and arsenic (Bennett, 1996; IMO, 1999). It was also thought that the tin could loose all organic groups, reverting to inorganic state and therefore becoming unavailable to biological organisms. Even so, TBT degradation rates, based on laboratory studies, seem longer in the environment than first expected (Burton *et al.*, 2006).

Tributyltin decay into less toxic metabolites, such as dibutyltin (DBT) and monobutyltin (MBT), occurs in water and sediment. Both compounds are far less toxic than TBT. Studies have attempted to identify the mechanism of TBT's biodegradation but whether if DBT is the primary degradation product followed by MBT and lastly a form of inorganic tin, is not yet completely known. Some studies showed this kind of mechanism whether others revealed a conversion of TBT directly to MBT (Dowson *et al.*, 1996). TBT breakdown has been observed to occur in several organisms ranging from bacteria to phytoplankton and birds. Even some bivalves manage to cause TBT breakdown. Animals that bioaccumulate less TBT, show more efficient TBT metabolism (Bryan and Gibbs, 1991; Takahashi *et al.*, 1999).

Organotins are not equally distributed in the environment as there is an evident relationship between TBT concentrations in water and sediments and the proximity of harbours, marinas and dockyards (Barroso *et al.*, 2000; Ohji *et al.*, 2007). In coastal waters, TBT tends to concentrate in the surface microlayer rather than being distributed in the water column. The typical half-life of TBT in ocean waters at 28ºC is 2.5 weeks (Stewart and de Mora, 1992). Regarding the sediments, the TBT half-life is less than 1 year in surface aerobic sediments (Bryan and Gibbs, 1991) but can be around 10 years for highly contaminated anoxic sediments. In view of the fact that much of the TBT is being buried in cold (below 10 ºC) oxygen-poor conditions, it is expected that TBT decay will take place over a number of decades (Dowson *et al.*, 1996). TBT can be regularly re-released into the environment due to maintenance dredging, which is often required to keep navigation channels, dock entrances and other shallow waters open (Langston and Pope, 1995; Dowson *et al.*, 1996).

The affinity of TBT for aquatic sediments, and the long term fate of this major persistent reservoir, extends the concern over this contaminant. The fact that benthic organisms may continue to accumulate TBT from this source together with the potential for desorption under certain conditions supports and emphasizes this idea (Langston and Pope, 1995; Takahashi *et al.*, 1999). Evidently, sediments cannot be thought as irreversible traps for organotins but, it may be incorrect to assume that particle-associated TBT is less likely to be bioaccumulated by infaunal organisms than the more mobile dissolved forms of the compound; hence, TBT accumulated in sediments may put benthic organisms at risk for many years to come (Langston and Pope, 1995).

While TBT is ubiquitous in the aqueous environment, an increasing number of studies have reported the occurrence of TPT mainly in biota (Hu *et al.*, 2006; Ohji *et al.*, 2007). Due to TBT restrictions in the late 1980s, more TPT has been used not only in AF paints but also as fungicide in agriculture activities (Hu *et al.*, 2006). The extent of the contamination by TPT and its ecotoxicological effects at the biochemical level are not well known (Fent *et al.*, 1998). Hu and co-workers (2006) confirm that TPT concentrations in fish, crabs and mussels from the Japanese Seto Inland Sea and the northwestern Mediterranean Sea are higher that TBT concentrations, although the proportion of TPT used is lower than TBT. This and other studies also suggest that TPT can be biomagnified in the marine aquatic food web (Hu *et al.*, 2006; Harino *et al.*, 2006). Conversely, TBT and other butyltins show no evidence of biomagnification throughout the food chain (Bryan and Gibbs, 1991; Dowson *et al.*, 1996; Takahashi *et al.*, 1999; Power and Keegan, 2001).

1.3 – Effects of TBT on marine organisms

The first severe biological effects from TBT use as AF agent on boats were discovered by a group of researchers at the University of Bordeaux (France). The oyster production almost ceased because of the elevated TBT concentrations in Arcachon Bay, which caused the development of oyster shell anomalies and low growth rates (Figure 2) (Alzieu*,* 1998; Alzieu*,* 2000). TBT would have affected the animal's ability to secrete calcium (Alzieu*,* 2000).

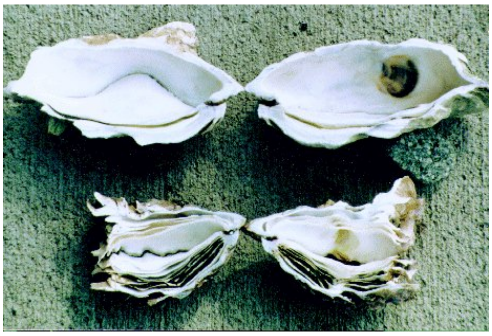


Figure 2 – Harmful effects on shell growth, induced by TBT exposure: top shell shows normal growth, the one on the bottom shows shell deformities (Canadian Department of Fisheries and Oceans, 1996).

Almost simultaneously, effects on other marine molluscs were reported and reduction of gastropods densities were described (de Mora, 1996). Blaber (1970) noted the occurrence of a penis like outgrowth behind the right tentacle on dogwhelk females. The term imposex was coined a year later, when Smith (1971), in a similar study, noticed similar abnormalities in the American mud snail *Nassarius obsoletus* females. This phenomenon was then described by the same author as the superimposition of male characteristics in gastropod females.

Smith (1971) carried out a large scale survey in order to understand what was causing this changes is *N. obsoletus* females. This study leaded to the hypothesis that imposex was somehow related with a substance that was arising from places where boating activity was higher - harbours and marinas. It was found that products containing TBT were the ones that were able to induce imposex (Smith, 1981).

Similar studies were made in order to assess if this same products responsible for causing imposex in females of *Nassarius obsoletus* were the same that were affecting *Nucella lapillus* females. Investigations regarding the decline of this common whelk also helped to support Smith's theory about TBT being the main contaminant inducing imposex in female snails (Bryan *et al.*, 1986). At last, the widespread occurrence of imposex phenomenon around the world was linked to TBT pollution.

TBT-induced imposex has been reported to occur in, at least, 150 prosobranch species worldwide (Tillmann *et al.,* 2001). In *Nucella lapillus*, females affected with imposex develop a penis and vas deferens that, in an advanced, stage can occlude the genital papillae blocking the egg duct and preventing the release of egg capsules. This fact renders female sterilization. The aborted capsules may build up and eventually they may cause the rupture of the capsule gland, which may kill the individual. Both processes might eventually lead to population decline (Bryan *et al.*, 1986). Growing juveniles are far more sensitive and may be sterilized at concentrations of only a few ng/L before reaching maturity (Bryan *et al.*, 1986). The imposex is thought to be of a permanent nature for the lifespan of an individual due to the irreversibility of the phenomenon (Gibbs *et al.*, 1987; Horiguchi, 2006).

Previous studies have shown that imposex is not entirely TBT specific. In 1988, Bryan and co-workers (1988) tested other compounds besides TBT - Tetrabutyltin (TTBT), dibutyltin (DBT), tripropyltin (TPrT) and triphenyltin (TPT) - in order to see if they could also induce imposex in the dogwhelk. TBT clearly developed imposex, TPrT and TTBT revealed slightly increases in female's penis length while the other two compounds did not produce any effect. Latter, in 1997, TPT was proved to clearly induce imposex in *Thais clavigera* (Küster) (Horiguchi *et al.,* 1997). Other authors have also proved that TPT is capable of promoting imposex but in a far less extent that TBT (Fent, 1996; Barroso, 2002; Nishikawa, 2006; Santos *et al.*, 2006). Nevertheless, TBT is the most significant promoter of imposex in the marine environment (Bryan *et al.*, 1988).

Studies trying to relate some metals with imposex were carried out but they showed no relationship between tissue burdens of arsenic, cadmium, copper, lead, zinc and silver with the masculinisation of the common dogwhelk (Bryan *et al.*, 1986).

Environmental TBT pollution is not restricted to coastal areas. Not only several species from the offshore were described to show imposex but also organotin accumulation on the offshore sediments has been demonstrated (Power and Keegan, 2001; Haller-Tjabbes *et al.*, 2003; Rato *et al.*, 2006; Gómez-Ariza *et al.*, 2006; Santos *et al.*, 2006).

Besides malformation in oysters and imposex in neogastropods other deleterious TBT effects were reported to occur in microalgae, polychaetes, crustaceans, bryozoans, echinoderms, tunicates and fish (Bryan and Gibbs, 1991). TBT has also been discovered in human blood and there is evidence that this compound can significantly inhibit human lymphocytes, reducing our ability to fight against cancers and viruses (Whalen *et al.*, 2000).

1.4 – Mechanisms underlying imposex induction

Imposex is one of the most well documented examples of endocrine disruption in the wildlife (WHO, 2002; Matthiessen and Gibbs, 1998). The mechanism(s) underlying imposex induction and/or development have not yet been fully clarified, however several hypotheses have been proposed.

Some authors demonstrated that the increased androgen levels in female gastropods, such as testosterone, could be associated with the aromatase inhibition by TBT (Spooner *et al.*, 1991; Bettin *et al.*, 1996). TBT might compete with the cytocrome P450 dependent aromatase preventing the conversion of androgens to estrogens, causing the build up of testosterone which induces imposex (Santos *et al.,* 2005; Oehlmann *et al.*, 2007). Other possible mechanism is proposed by Ronis and Mason (1996) according to which an inhibition of the excretion of androgens' sulphate conjugates by TBT may occur causing a built up of androgens. Organotin compounds can alter both esterification of testosterone and its sulfonation as reported by Janer *et al.* (2005).

Féral and Le Gall (1983) suggested that the TBT-induced imposex in *Ocenebra erinacea* was related to the release of a neuroendrocrine factor (Penis Regression Factor), by the cerebropleural ganglia, which was responsible for the suppression of penis formation in females. In no affected females, this factor would prevent the neurohormones secretion by the pedal ganglia that controlled penis growth. TBT would inhibit the release of this factor resulting in imposex development. In addition, Oberdorster and McClellan-Green (2000) reported that the increase in a neuropeptide level - the APGWamide - caused by TBT could induce imposex in *I. obsoleta*. Recently, Nishikawa and co-workers (2004) suggested the involvement of the Retinoid X Receptor (RXR) in the induction, differentiation and growth of male genital organs in *N. lapillus* female gastropods. This hypothesis shows that gastropods have a functional homologue of RXR to which both TBT and TPT are high-affinity ligands. They also demonstrated that the RXR natural ligand 9-cis retinoic acid also caused imposex development in female rock shells (Nishikawa, 2006; Horiguchi, 2006). A recent report also demonstrated that imposex induction is mediated trough RXR in *N. lapillus* (Castro *et al.*, 2007).

The mollusc's hormone system is perhaps the most diverse of all invertebrate phyla as it can differ between classes and inside the same class (Oehlmann *et al.*, 2007). Many unanswered questions still persist, some authors point out the fact that the existence of vertebrate-type aromatase in gastropods is not clear and was never isolated (Horiguchi, 2006). In this way further studies on TBT and TPT mechanism of action for the induction and development of imposex should be carried on.

1.5 – Habitat, distribution and biology of *Nucella lapillus*

The common dogwhelk, *Nucella lapillus*, is a predatory gastropod mollusc, existing on most rocky shores communities throughout the littoral zone of the North Atlantic (Crothers, 1980) from the Arctic to the Algarve (south Portugal) in the east, and from Greenland to Long Island in the west, including Iceland (Berry and Crothers, 1974) (Figure 3).

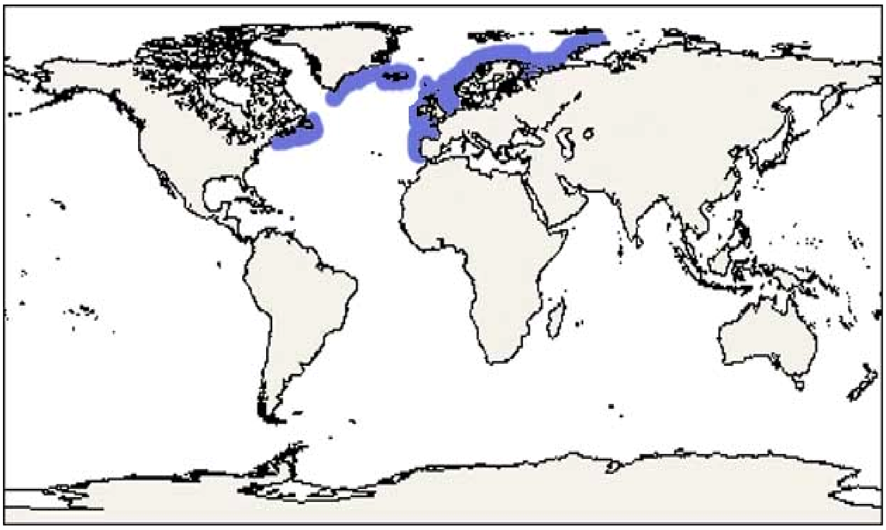


Figure 3 – Global geographical distribution of the common dogwhelk, *Nucella lapillus* (L.) (Tyler-Whalters, 2003).

The appearance of dogwhelks' shell is extremely variable and depends on various factors (Crothers, 1983). The general shape consists of a pointed spire, dominated by the last whorl. The presence of the operculum closes the shell when the animal is contracted and offers some protection against predation and dissection when the tide is out (Fish and Fish, 1996).

Shell colouration may vary between white, brown shading to black, mauve grading to pink, yellow shading to orange and rarely true orange, pink or black. They can also exhibit a diversity of banded forms, with a combination of un-banded, thin or thick banded (Figure 4) (Crothers, 1985; Fish and Fish, 1996; Tyler-Whalters, 2003).

Figure 4 – Diversity of colours and patterns in the shell of the dogwhelk *Nucella lapillus*.

These animals are inhabitants of rocky shores from the mid shore downwards. Rarely present in the sub littoral, the common dogwhelk may be abundant in areas exposed to extremely strong tidal stress as well as sheltered shores. They are an important intertidal predator that preys essentially on barnacles and mussels but may also prey on cockles, other bivalves and gastropods (Crothers, 1985; Fretter and Graham, 1994; Gibbs, 1999). These animals are able to bore into their prey's shells but can also enter their body by a simple thrust of the proboscis. This last method is energetically more economic but reflects the predator's strength. Boring throughout a mussel or a barnacle shell may take up to three days and is made by a combination of mechanical and chemical means. Dogwhelks secrete enzymes to soften the shell, which can then be bored by their radula (Fish and Fish, 1996). They search for prey only while covered by the tide but once the handling time of the prey can range from several hours until a few days, foraging exposes this animals to a range of potential hazards that includes desiccation, predation and dislodgement by waves (Hughes and Taylor, 1997).

In terms of physiographic preferences, dogwhelks can be found on open coasts or in enclosed ones, on Straits or Estuaries and others. All these places have different tidal strengths to which dogwhelks are adapted to. The adults can be found on a multiplicity of different habitats. On sheltered shores, we can find them on open rocks and boulders, overhang or in rock pools. On the other hand, on exposed shores, adults can be found mainly in crevices and under or beneath boulders. We can also see adult dogwhelks attached to artificial objects such as metal and woods for example on the base of piers (Tyler-Whalters, 2003).

Depending on their habitat, wave exposure, prey type, and starvation, growth rates may be different. Sheltered shore populations grow faster than wave exposed shore ones, resulting in larger and more elongated shells (Crothers, 1985; Etter, 1989). Etter (1996) concluded that growth was determined by environmental factors and significantly depressed by wave exposure since it reduces feeding and handling time. Dogwhelks from wave exposed shores tend to have shorter, squatter shells than those from sheltered shores, which are more elongated. A progression from squat to elongated form is seen with the decreasing of the wave exposure (Crothers, 1985). In this way, shell shape, shell thickness and relative size of the aperture is known to vary with wave exposure.

There are possible reasons to explain the relationship between wave exposure and shell parameters: dogwhelks with short squat shells offer less resistance to wave action and water flow, exhibiting a larger aperture and larger foot, which increases their adhesion to the substratum surface (Crothers, 1985; Etter, 1989). Instead they are more prone to predation as they have rounded shapes that make them easier to swallow by gulls and ducks. They are also susceptible to oystercatchers and crabs because they are not able to hide completely inside their shell. The ones with longer, elongated shells have a relatively smaller foot but can hold a significantly greater volume of water within its mantle cavity when emersed and are more tolerant to desiccation (Crothers, 1992; Kirby *et al.*, 1994). This characteristic is important when the shore is sheltered and water availability can be low when the tide is out. Moreover, tall and narrow shells are not easily handled by crabs and the thick outer lip is difficult to crack open (Fish and Fish, 1996).

Some studies suggested that populations occurring in shores with different kinds of exposure may reveal genetic differences namely in the number of chromosomes. In 1957 a study carried out by Staiger in Roscoff - France, revealed two distinct races inside the species *N. lapillus*: one occurring mostly in exposed shores with 13 pairs of chromosomes and other more characteristic of sheltered situations with 18 pairs (Fretter and Graham, 1994). Fretter and Graham (1994) suggest that some differences between dogwhelks can be genetically based, even if these differences between populations do not always occur. It is unclear if the variation represents genetic differentiation or phenotypic plasticity but other studies showed that transplantations of adults from exposed to sheltered shores, produced young animals that developed a close resemblance to sheltered shore morphology. Genotype-environment interactions are obvious and both are likely to influence the shell shape in this species (Hughes and Taylor, 1997).

Dogwhelks show aggregative behaviour throughout most of their life cycle but above all in the summer (from May to October). Aggregations of 20 - 500 individuals of diverse ages may be formed on the open rock surfaces (Feare, 1971; Crothers, 1985). In the winter, individuals aggregate in crevices and pools, most probably to avoid dislodgement, since they have difficulty re-attaching in cold weather. Aggregations that are formed during the winter, may structure into pre-breeding and breeding aggregations (Feare, 1971; Crothers, 1985).

Nucella lapillus is a gonochoristic neogastropod with internal fertilization whose equivalent of the larval stage is completed within the egg capsule and the young emerge at the crawling stage complete with shells, like an adult in miniature (Crothers, 1977; Etter, 1996). The egg capsules are vase shaped, about 8mm high, usually yellow, and found attached to hard substrata, crevices and under overhangs (Figure 5). Recently hatched juveniles are found at the same level as their capsules, and prefer the protection of crevices, boulders, pools, and the empty cases of barnacles, avoiding open rocks (Crothers, 1977; Crothers, 1985; Gibbs, 1999).

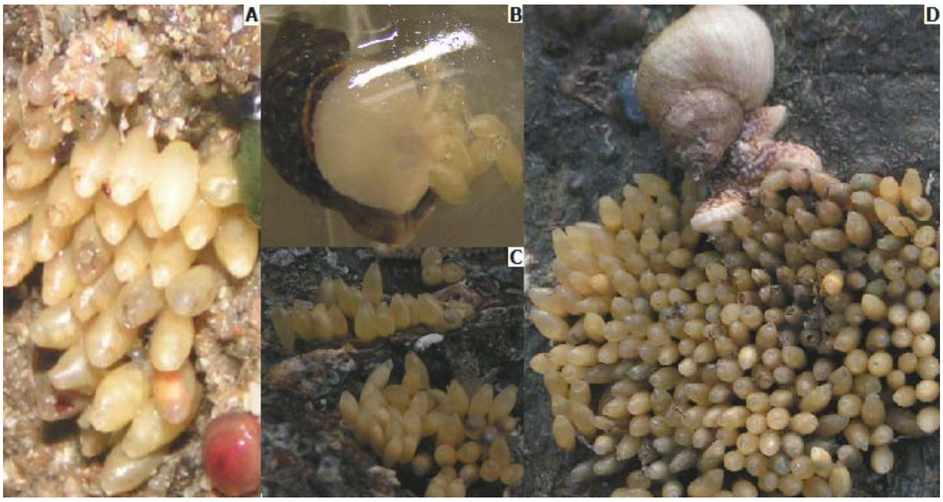


Figure 5 – Capsules of the dogwhelk *Nucella lapillus* (L.). A – Capsules found inside a crevice in Portugal; B – Dogwhelk laying capsules under laboratory conditions; C and D – Capsules found at Menai Bridge, Wales.

A newly deposited capsule may contain up to 600 eggs but only a few (15-30) will complete its development while the others may act as food for embryos. Stage of development of the egg capsule can be easily recognized. Newly deposited capsules seem to have a more intense and homogeneous yellow coloration (Figure 5 – B), then, as embryos remain inside the capsule for 3 or 4 months, a different texture can be seen inside the capsules (Figure 5 – A and C) and, as the time goes by, capsules start to open the apical hole (as it can be seen in the centre of Figure 5 – D) and the juveniles emerge crawling (Fish and Fish, 1996; Gibbs, 1999).

Crothers (1977) states that, at the time that maturity is reached, the shell no longer increases in size and the sharp growing edge of the lip becomes worn thickened and sometimes toothed. Etter (1996) refutes this idea by saying that under benign conditions whelks continue to grow after reaching maturity but more slowly. Even though, both authors agreed on saying that dogwhelks reach maturity after about 3 years of life. Disturbance or interruptions of growth, earlier in life, probably caused by starvation or parasitism may result in additional rows of teeth (Crothers, 1985). Dogwhelks can also be hosts for trematode parasites of sea birds, *Parorchis acanthus* and *Lepocreadium* sp (Proud, 1994).

This species provides an excellent subject for studies as it is a conspicuous, widely distributed, common, harmless to man, long-living and with no importance commercial value. *Nucella lapillus* lacks a dispersive pelagic larval phase and, because of that, forms discrete populations of related individuals (Gibbs, 1999).

1.6 – *Nucella lapillus* as a bioindicator

The use of biomarkers and biological indicators for environmental monitoring has been playing a leading role in environmental management because of their ecological relevance (Axiak *et al.*, 2003). Several species have been proposed and validated for biomonitoring TBT contamination through the imposex response. Biomonitoring may overcome some difficulties related to the chemical measurement of TBT environmental concentrations: the assessments of large temporal variations of this compound at a fixed location and also the fact that TBT can be biologically effective at concentrations below the limit of detection (Barroso *et al.*, 2000). Besides, it is a low cost technic with a biological meaningful measure of organotin contamination (Barroso *et al.*, 2000). Even though, chemical analysis are strongly recommended in order to provide confirmation of the relation between imposex and organotin concentrations (OSPAR, 1998).

Nucella lapillus is considered one of the most sensitive bioindicators of TBT pollution by the Oslo and Paris Commission (OSPAR) and has been largely used as a bioindicator of the TBT pollution along the coasts of UK (Bryan *et al.*, 1986; Gibbs *et al.*, 1987; Gibbs and Bryan, 1986; Minchin *et al.*, 1995; Evans *et al.*, 1996; Minchin *et al.*, 1997; Miller *et al.*, 1999; Evans *et al.*, 2000; Birchenough, 2002), France (Huet *et al.*, 2004), Spain (Ruiz *et al.*, 1998), Portugal (Barroso and Moreira, 2002; Santos *et al.*, 2000; Santos *et al.*, 2002; Galante-Oliveira *et al.*, 2006), Germany and other northern countries (Oehlmann *et al.*, 1998; Harding *et al.*, 1999).

1.6.1 – Sex Determination

Differences between sexes cannot be assessed by the analysis of shell characters; however, it is possible to distinguish males from females without sacrificing them as long as they are narcotized. For a complete imposex examination the body must be extracted from the shell and, in this way, sexes may be reliably distinguished, by the presence of two easily recognisable features in the females: the capsule gland and the sperm ingesting gland (Figure 6). Both features are absent in males, whilst the presence of the prostate gland, which has a mustard yellow coloration, occurs in the same body localization than the female's capsule gland that exhibit a much paler colour, normally appearing creamy white and being a bulbous structure. The sperm ingesting gland has a dark brown coloration and is situated immediately posterior to the capsule gland. This gland is known as the "brown gland" and appears to be the most distinctive and easy recognisable characteristic. The absence of a similar structure in the males makes sexing a relatively easy task (Fretter and Graham, 1994). In an older age both prostate and capsule gland can become brownish, in this way the sperm ingesting gland remains the easiest character to distinguish both sexes.

Only immature individuals who have shell lengths <8mm (probably aged less than 6 months) reveal some difficulties in the sex differentiation procedure (Gibbs *et al.*, 1987). This happens for the reason that the sperm ingesting gland may not be clearly developed.

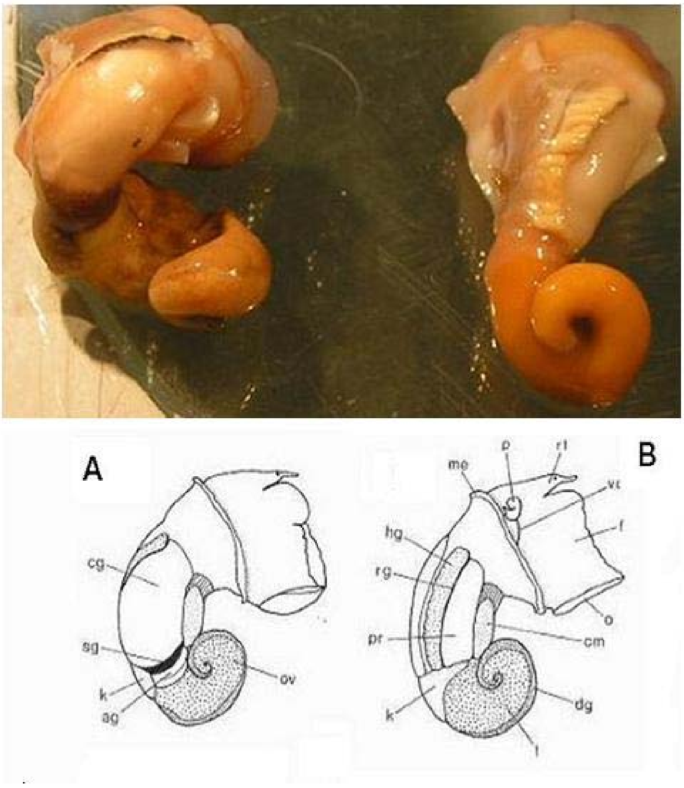


Figure 6 – *Nucella lapillus.* External features of mature female (A) and male (B) after shell's removal both in the upper picture and in the schematic figure (below). Abbreviations: ag – albumen gland; cg – capsule gland; cm – columella muscle; dg – digestive gland; f – foot; hg – hypobranchial gland; k – kidney; me – mantle edge; o – operculum; ov – ovary; p – penis; pr – prostate; rg – rectal gland; rt – right tentacle; sg – sperm ingesting gland; t – testis; vd – vas deferens (Gibbs *et al.*, 1987).

1.6.2 – Imposex response in *Nucella lapillus*

Imposex is the superimposition of male genitalia onto the female (see point 1.3). Initially, Blaber (1970) measured the degree of imposex calculating the proportion of penis-bearing females in the population. Before the implementation of legislative measures prohibiting the utilization of TBT and in the following years, it was rare to find populations were the majority or even all females did not bear a penis. In severally affected females, the penis length approached that of the male (Gibbs *et al.*, 1987).

As a result, other methods were required and the expression of imposex started to be assessed by recognizing stages in the vas deferens development and in the penis size. These procedures were initially developed by Smith (1971) in *N. obsoletus*, but it has now been used in several gastropods worldwide. In *N. lapillus*, imposex has been mainly quantified by four indices: Relative Penis Size Index (RPSI), Vas Deferens Sequence Index (VDSI) (Gibbs *et al.*, 1987), Female Penis Length Index (FPLI) and Percentage of females affected by imposex (%I). The percentage of sterilized females (%S) may be also used at highly polluted sites.

Female Penis Length (FPL)

In *N. lapillus*, penis size is related with the body size. Bigger dogwhelks are known to have bigger penis. Generally, even when imposex was affecting more severely dogwhelk's populations, the average length of the female penis was observed to be smaller than those of the males. The penis is situated immediately behind the right tentacle and is very easy to see. No attempt to straighten the penis should be made since it presents itself as a relatively stout and immobile structure. Early penis development in females appears as a ridge (Gibbs *et al.*, 1987).

Measurement of the penis length in *N. lapillus* both in males (Male Penis Length Index – MPLI) and females (Female Penis Length Index – FPLI) is a simple and quick procedure. Normally, a cut following the line of the rectal gland is made, since this allows the pinning back of the mantle thus exposing the external genital features of both sexes fully. However, this procedure is not crucial when making penis size assessment. Its length is measured from the base – the junction with the body wall behind the right tentacle – to its tip, using a dissecting microscope equipped with a graduated eyepiece. Whenever abnormalities are found (bi or even trifurcate penis) then the structure measured is the longest one (Gibbs *et al.*, 1987). Penis length should be measured as soon as possible after the removal from the shell and without narcotisation that would lead to an extension of the penis (Minchin and Davies, 1999).

Relative Penis Size (RPS)

Relative penis size is defined as the mean bulk of the female penis expressed as a percentage of the mean bulk of the male penis from the same population, where the bulk of the penis is calculated as the cube of its length (Gibbs *et al.*, 1987).

$$\text{RPSI} = \frac{(\text{mean length female penis})^3}{(\text{mean length male penis})^3} \times 100$$

Whilst mean length comparisons would be the simplest to achieve, they give an inadequate representation of the differences between penis sizes in different sampled sites (Gibbs *et al.*, 1987). In view of the fact that body size varies among sites to the proportion of exposed and sheltered populations (Crothers, 1985), FPL alone could be a poor indicator of TBT levels. That is why comparisons were made using the mean bulk (weight or volume), in order to remove body size as a source of variation when calculating the RPSI. This way, the difference between RPSI from different sites is assumed to be due only to TBT exposure and not any other factor. However, when comparisons are being made between years, on the same site and with no significant differences in shell heights, FPLI can also be a good indicator of TBT levels.

There are two major disadvantages with this index. The first one is related to the fact that RPSI does not show a direct relation with TBT levels in the water and secondly, in populations exposed to high levels of TBT contamination, the males often develop growths on or around the penis which can lead to an overestimation of the penis length. Consequently, the RPSI will be smaller leading to an underestimation of the degree of contamination (Bryan *et al.*, 1986).

The major advantage of this index, relatively to VDSI, is that repeatability between observers is easily achieved (Gibbs *et al.*, 1987), allowing a consistent comparison between different authors.

Vas Deferens Sequence (VDS)

The vas deferens development is generally assessed using the Vas Deferens Sequence Index (VDSI). VDS evaluation is relatively complex as it is necessary to make an examination of the penis, the vas deferens and the genital papilla. A 3 stage squeme was firstly proposed by Bryan and Gibbs (1986) in order to use the VDS not only as an indicator of TBT pollution but also to evaluate the reproductive capacity of the population. Later, this was revised and extended to a six point scale by Gibbs *et al.* (1987). This protocol has been used ever since to categorise imposex development from stage 0 (normal) to stage 6 (grossly affected). Development of imposex is divided into the following defined stages (Figure 7 and Table 1):

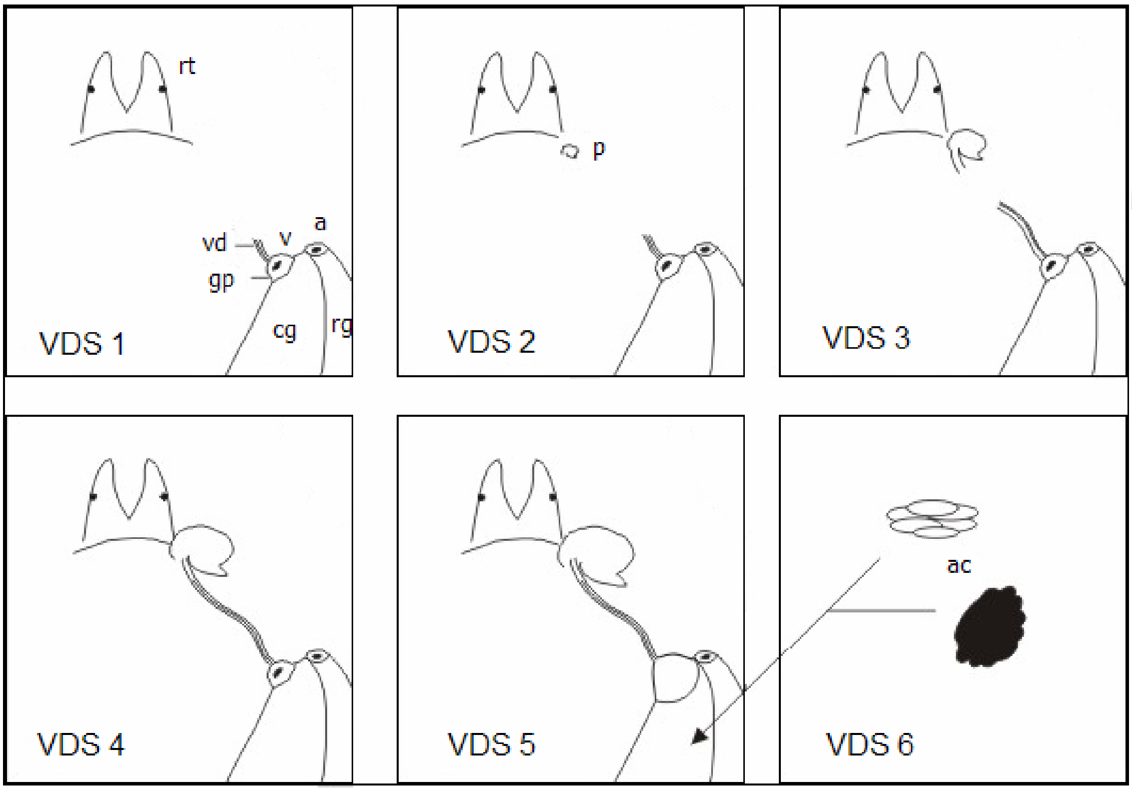


Figure 7 – The six stages of the Vas Deferens Sequence in the development of imposex in the common dogwhelk *Nucella lapillus* from initialization (stage 1) to sterilization (stage 5) and ultimately the accumulation of aborted egg capsules in the capsule gland (stage 6). Abbreviations: a – anus; ac – aborted capsules; cg – capsule gland; gp – genital papillia; p – penis; rg – rectal gland; rt – right tentacle; v – vulva; vd – vas deferense (Gibbs *et al.*, 1987).

Table 1 – Stages in imposex development (Gibbs *et al.*, 1987).

Stages	Imposex development
0	The unaffected female with no apparent male characters. The genital papilla and the vulva are clearly visible with no development of the vas deferens tissue.
1	An infolding of the mantle cavity epithelium in the ventral area next to the genital papilla marks the first development of the vas deferens.
2	Initiation of penis development by the formation of a ridge behind the right tentacle.
3	The ridge becomes recognisable as a small penis and development of the vas deferens from the base of the penis begins.
4	The two sections of vas deferens, started at the genital papilla and at the base of the penis, become fused into a continuous tube. The penis is larger and closer to the male penis size and shape.
5	The proliferation of the vas deferens tissue on the genital papilla (hyperplasia) results in the occlusion of the vulva, and the genital papilla becomes displaced, constricted or no longer visible.
6	The lumen of the capsule gland contains the material of aborted capsules; this material may comprise a single capsule or several to many that are compressed together to form a translucent or light to dark brown mass.

1.7 – Legislation and alternatives to TBT

The legislation banning the use of TBT on boats less than 25m was firstly introduced in France in 1982, following the collapse of their oysters industry (Alzieu, 2000). The UK government was slower to react but in 1986 concentrations of tin in AF paints were restricted. Nevertheless, it was not until 1987 that legislation came into force announcing a prohibition on a large scale on the application of TBT AF paints in boats less than 25m in length and on supply of TBT AF paints and its application on nets and fishing cages used in fishing farms (Abel *et al.*, 1987).

Only in 1989 the European Union published the Directive 89/677/CEE that was amending for the eighth time the original Directive 76/769/EEC related to restrictions on the marketing and use of certain dangerous substances, banning TBT from boats smaller than 25m. In 2001, the International Maritime Organization (IMO) adopted the "International Convention on the Control of Harmful Antifouling Systems on ships". This resolution called for a global prohibition on the application of OTs based AF paints after the 1st of January of 2003 and a complete ban on its presence in ship hulls from the 1st of January of 2008 (MEPC, 2001). However, the convention could just enter into force 12 months after 25 States, representing 25% of the world's merchant shipping tonnage, have ratified it. Until then, the legal effect of the 1st January 2003 would be suspended and, during the time elapsed till the entry into force date, even the countries that ratified the document could not apply the new restrictions to foreign ships calling into their ports. Meanwhile, based on an IMO resolution invinting Member States to implement the agreement as a matter of urgency, the European countries banned the marketing and use of tin organic compounds, by the Directive 2002/62/EC, and adopted the EC Regulation 782/2003 on OTs coatings on Member States national merchantile fleets, and on ships operating under their authority, from the 1st of July 2003.

The entry into force date of the IMO convention was finnaly met on 17 September 2007 with the 25th state ratification, representing a total 38.09% of the required world's tonnage. As a result, the total ban was stated for 17 September 2008 (www.imo.org).

Despite these restrictions there are still new inputs of TBT into the environment due to the fact that large foreign vessels over 25m in length are still legally allowed to use AF paints until 2008 and also because of TBT's slow degradation rate. On the same hand, illegal use of paints, despite efforts to reduce them, seem to be a reality so far with boat proprietors continuing to use these paints because of the lack of viable alternatives (Proud, 1994).

As a consequence of the TBT ban, coating manufactures had to devise new ways of producing tin-free alternatives with the same performance of TBT – SPC paints through the development of new matrices and/or the use of non-persistent co-biocides (Howell and Behrends, 2006). Nowadays, the literature available from the global coatings' manufacture has indicated that there are two major types of alternative AF paints: those that have hydrating binders – known as ablative or solid matrix coatings and the ones that mimic TBT-SPC coatings by having hydrolysing binders. In either case, copper is the biocide of choice for present day AF paints since it is a naturally occurring element and has not known long-term harmful effects on humans. However, if further studies are not conveniently done, it might reach concentrations that can seriously impact the environment (Srinivasan and Swain, 2007).

These products may also contain organic biocides to boost the efficacy of the formulations by inhibiting primary growth of copper resistant fouling organisms like diatoms, cyanobacteria and some higher algae members of the phylum Chlorophyta (Jelic-Mrcelic *et al.*, 2006). For many of these compounds that act as active ingredients in AF products, the available literature on effects on the aquatic life is limited but are now starting to reveal nasty side effects. Some of these booster biocides are Zinc Pyrithione, Zineb, Sea Nine 211, Irgarol and Diuron (Karlsson *et al.*, 2006; Harino *et al.*, 2006). Countries like UK, Sweden and Denmark have already banned the use of Diuron and Irgarol since laboratory studies with low concentrations of these compounds showed negative effects on aquatic organisms (Karlsson *et al.*, 2006). However, some studies relating OTs with these kinds of biocides were done and revealed that contamination by TBT is still a more serious problem than contamination by booster biocides, even after so many years have passed since the ban of TBT-based AF paints in small boats and other marine structures. This also suggests that the ecotoxicological risk associated with organotins has not diminish (Biselli *et al.*, 1999; Harino *et al.*, 2006).

Biocide-free coatings such as: non-stick paints, silicone-based fouling release system, ceramic-epoxy coatings and self-polishing biocide-free paints, are also becoming available (Waterman *et al.*, 2005; Srinivasan and Swain, 2007). Waterman and co-workers (2005) observed that silicones displayed no toxic properties but an improvement of its environmental characteristics can be achieved by the addiction of copper compounds instead of OTs. These paints are not as long lasting as TBT based ones so, there have been a new interest in the development of other alternatives.

Plenty of studies on natural products or synthesized analogues are now arising in order to find a substitute to TBT-based AF coatings. Usually, these compounds act enzymatically by interfering with the metabolism or dissolving adhesive materials of the fouling organisms. Qi and co-workers (2006) claimed that some natural products isolated from the gorgorian coral *Junceella juncea* have AF properties. This approach to the prevention of fouling is, however, far from commercial exploitation.

Furthermore, other system called MAGPET (marine growth prevention by electrolysis technology) as been developed by a Japanese company. This system makes an electrical current pass through a conductive coating which results in electrolytic action though the surrounding seawater producing acid ions around the ship hull preventing the settlement of marine organisms (Proud, 1994). In the opinion of some authors like Readman (2006), the production of engineered highly reactive nano-particles would afford a high-tech alternative to AF in the future.

Moreover, an effective risk assessment should be made to guarantee that any compound used in AF paints is not more or as dangerous as those who are being replaced, and that the legacy of environmental damage created by TBT is not repeated (Thomas, 2002).

1.8 – Rationale of the present dissertation

The present study is constituted by four chapters. Initially, in Chapter 1, is given a general introduction that provides a brief characterization of the history of organotin pollution, its effects on the organisms and the use of *N. lapillus* as a bioindicator of this kind of pollution. In Chapter 2, it is described an experiment that proves the cause-effect relationship between TBT and imposex and compares the response of two populations of dogwhelks which seemed to present different sensibilities to TBT. Chapter 3 presents the spatial distribution of dogwhelks' imposex and organotin contamination throughout North Wales and analysis the temporal evolution of TBT pollution in the area from 1987 to 2006. In Chapter 4, it is presented a succinct conclusion of this work. Finally, it is provided an Annex with a brief description of each sampling site and dogwhelk abundance in order to better characterize the actual status of the seashore and associated dogwhelks populations to be used as a baseline for future monitoring surveys.

1.9 – References

Abel R, Hathaway RA, King NJ, Vosser JL, Wilkinson TG (1987). Assessment and regulatory actions for TBT in the UK, Oceans '87. Conference Record 4: 1314 - 1319.

Alzieu C (1988). Tributyltin: case study of a chronic contaminant in the coastal environment. Ocean & Coastal Management 40: 23 - 36.

Alzieu C (2000). Impact of Tributyltin on Marine Invertebrates. Ecotoxicology 9: 71 - 76.

Appel K (2004). Organotin compounds: Toxicokinetics Aspects. Drug Metabolism Reviews 36: 763 - 786.

Axiak V, Micallef D, Muscat J, Vella A, Mintoff B (2003). Imposex as a biomonitoring tool for marine pollution by tributyltin: some further observations. Environment International 28 (8): 743 - 749.

Barroso CM, Moreira MH (2002). Spatial and temporal changes of TBT pollution along the Portuguese coast: inefficacy of the EEC directive 89/677. Marine Pollution Bulletin 44: 480 - 486.

Barroso CM, Moreira MH, Gibbs PE (2000). Comparison of imposex and intersex development in four prosobranch species for TBT monitoring of a southern European estuarine system (Ria de Aveiro, NW Portugal). Marine Ecology Progress Series 201: 221 - 232.

Barroso CM, Reis-Henriques MA, Ferreira MS, Moreira MH (2002). The effectiveness of some compounds derived from antifouling paints in promoting imposex in *Nassarius reticulatus*. Journal of the Marine Biological Association of the United Kingdom 82: 249 - 233.

Bennett RF (1996). Industrial manufacture and applications of tributyltin compounds. In de Mora SJ (ed) Tributyltin: case study of an environmental contaminant. No.8, Cambridge Environmental Chemistry Series, Cambridge University Press, Cambridge, pp 21 - 61.

Berry RJ, Crothers JH (1974). Visible variation in the dog-whelk, *Nucella lapillus*. Journal of Zoology of London 174: 123 - 148.

Bettin C, Oehlmann J, Stroben E (1996). TBT – induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgolander Meeresuntersuchungen 50: 299 - 317.

Birchenough AC, Evans SM, Moss C, Welch R (2002). Re-colonisation and recovery of populations of dogwhelks *Nucella lapillus* (L.) on shores formerly subject to severe TBT contamination. Marine Pollution Bulletin 44: 652 - 659.

Biselli S, Bester K, Huhnerfuss H, Fent K (1999). Concentrations of the Antifouling Compound Irgarol 1051 and of Organotins in Water and Sediments of German North and Baltic Sea Marinas. Marine Pollution Bulletin 40 (3): 233 - 243.

Blaber SJM (1970). The occurrence of penis-like outgrowth behind the right tentacle in spent females of *Nucella lapillus*. Proceedings of the Malacological Society of London 39: 231 - 233.

Bryan GW, Gibbs PE (1991). Impact of low concentrations of Tributyltin (TBT) on Marine Organisms: a Review. In Newman MC, McIntosh AW (eds) Metal ecotoxicology: concepts and applications. Lewis Publishers, Ann Arbor, Boston, pp 323 - 361.

Bryan GW, Gibbs PE, Burt GR (1988). A comparison of the effectiveness of Tri-n-Butyltin chloride and five other organotin compounds in promoting the development of imposex in the dog-whelk, *Nucella lapillus*. Journal of the Marine Biological Association of the United Kingdom 68: 733 - 744.

Bryan GW, Gibbs PE, Burt GR, Hummerstone LG (1987). The effects of tributyltin (TBT) accumulation on adult dog–whelks, *Nucella lapillus*: Long-term field and laboratory experiments. Journal of the Marine Biological Association of the United Kingdom 67: 525 - 544.

Bryan GW, Gibbs PE, Hummerstone LG, Burt GR (1986). The decline of the gastropode *Nucella lapillus* around South-West England: evidence for the effect of tributyltin from antifouling paints. Journal of the Marine Biological Association of the United Kingdom 66: 611 - 640.

Burton ED, Phillips IR, Hawker DW (2006). Tributyltin partitioning in sediments: Effect of aging. Chemosphere 63: 73 - 81.

Canadian Department of Fisheries and Oceans (1996) - [cited 25/06/05]. Available from: http://www-sci.pac.dfo-mpo.gc.ca/mehsd/products/tbt/tbt_e.htm

Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J, Nakanishi T, Reis-Henriques MA, Santos MM (2007). Imposex induction is mediated through the Retinoid X Receptor signalling pathway in the neogastropod *Nucella lapillus.* Aquatic Toxicology 85: 57 - 66.

Champ MA (2000). A review of organotin regulatory strategies, pending actions, related costs and benefits. The Science of the Total Environment 258: 21 - 71.

Crothers JH (1977). Some observations on the growth of the common dogwhelk, *Nucella lapillus* (Prosobranchia: Muricacea) in the laboratory. Journal of Conchiology 29: 157 - 162.

Crothers JH (1980). Further observations of the common dog-welk, *Nucella lapillus* (L.), in the laboratory. Journal of Molluscan Studies 46: 181 - 185.

Crothers JH (1983). Some observations on shell-shape variation in North American populations of *Nucella lapillus* (L.). Biological Journal of the Linnean Society 19: 237 - 274.

Crothers JH (1985). Dog-whelks: an introduction to biology of *Nucella lapillus* (L.). Field studies 6: 291 - 360.

Crothers JH (1992). A re-evaluation of shell shape variation in Shetland dog-whelks, *Nucella lapillus* (L.) and their use as biological exposure indicators. Journal of Molluscan Studies 58: 315 - 323.

Dowson PH, Bubb JM, Lester JN (1996). Persistence and Degradation Pathways of Tributyltin in Freshwater and Estuarine Sediments. Estuarine, Coastal and Shelf Science 42: 551 - 562.

Etter RJ (1989). Life history variation in the intertidal snail *Nucella lapillus* across a wave-exposure gradient. Ecology 70 (6): 1857 - 1876.

Etter RJ (1996). The effect of wave action, prey type, and foraging time on growth of the predatory snail *Nucella lapillus* (L.). Journal of Experimental Marine Biology and Ecology 196: 341 - 356.

Evans SM, Evans PM, Leksono T (1996). Widespread Recovery of Dogwhelks, *Nucella lapillus* (L.), from Tributyltin Contamination in the North Sea and Clyde Sea. Marine Pollution Bulletin 32 (3): 263 - 269.

Evans SM, Kerrigan E, Palmer N (2000). Causes of Imposex in the dogwhelk *Nucella lapillus* (L.) and its use as a Biological Indicator of Tributyltin Contamination. Marine Pollution Bulletin 40 (3): 212 - 219.

Feare CJ (1971). The Adaptive Significance of Aggregation Behaviour in the dogwhelk *Nucella lapillus* (L.). Oecologia 7: 117 - 126.

Fent K (1996). Ecotoxicology of organotin compounds. Critical Reviews in Toxicology 26: 1 - 117.

Fent K, Woodin BR, Stegeman JJ (1998). Effects of triphenyltin and other organotins on hepatic monooxygenase system in fish. Comparative Biochemistry and Physiology Part C 121: 277 - 288.

Féral C, Le Gall S (1983). The influence of a pollutant factor (TBT) on the neurosecretory mechanism responsible for the occurrence of a penis in the females of *Ocenebra erinacea*. In Lever L, Boer HH (eds) Molluscan Neuro-Endocrinology. North Holland Publishing, Amsterdam, pp 173 - 175.

Fretter JD, Graham A (1994). The dog whelk, *Nucella lapillus*. In Fretter V, Graham A (eds) British Prosobranch molluscs, their functional anatomy and ecology. Revised and updated edition No. 161, The Ray Society of London, London, pp 707 - 711.

Fish JD, Fish S (1996). In Fish JD, Fish S (eds) A Student´s Guide to the Seashore. 2nd edition, Cambridge University Press, Cambridge, pp 213 - 257.

Gadd GM (2000). Microbial interactions with tributyltin compounds: detoxification, accumulation, and environmental fate. The Science of the Total Environment 258: 119 – 127.

Galante–Oliveira S, Langston WJ, Burt GR, Pereira ME, Barroso CM (2006). Imposex and organotin body burden in the dog-whelk (*Nucella lapillus* L.) along the Portuguese coast. Applied Organometallic Chemistry 20: 1 - 4.

Gibbs PE (1999). Biological effects of contaminants: Use of imposex in the dogwhelk (*Nucella lapillus*) as a bioindicator of tributyltin pollution. ICES Techniques in Marine Environmental Sciences 24: 1 - 29.

Gibbs PE, Bryan GW (1986). Reproductive failure in populations of dogwhelks, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints. Journal of the Marine Biological Association of the United Kingdom 66: 767 - 777.

Gibbs PE, Bryan GW (1987). The Paints and the demise of the dog whelk, *Nucella lapillus* (Gastropoda). Journal of the Marine Biological Association of the United Kingdom 19: 1482 - 1487.

Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1987). The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. Journal of the Marine Biological Association of the United kingdom 67: 507 - 523.

Gómez–Ariza JL, Santos MM, Morales E, Giráldez I, Sánchez-Rodas D, Vieira N, Kemp JF, Boon JP, Ten-Hallers-Tjabbes CC (2006). Organotin contamination in the Atlantic Ocean off the Iberian Peninsula in relation to shipping. Chemosphere 64: 1100 - 1108.

Goldberg ED (1986). TBT: an environmental dilemma. Environment 28: 17 - 44.

Harding MJC, Davies IM, Bailey SK, Roder GK (1999). Survey of imposex in dogwhelks (*Nucella lapillus*) from North Sea Coasts. Applied Organometallic Chemistry 13: 521 - 538.

Harino H, Ohji M, Wattayakorn G, Arai T, Rungsupa S, Miyazaki N (2006). Occurrence of Antifouling Biocides in Sediments and Green Mussels from Thailand. Environmental Contamination and Toxicology 51: 400 - 407.

Horiguchi T (2006). Masculinization of Female Gastropod Mollusks Induced by Organotin Compounds, Focusing on Mechanism of Actions of Tributyltin and Triphenyltin for Development of Imposex. Environmental Sciences 13 (2): 77 - 87.

Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997). Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. Environmental Pollution 95: 85 - 91.

Howell D, Behrends B (2006). A methodology for evaluating biocide release rate, surface roughness and leach layer formation in a TBT-free, self polishing antifouling coating. Biofouling 22 (5): 303 - 315.

Hu J, Zhen H, Wan Y, Gao J, An W, An L, Jin F, Jin X (2006). Trophic Magnification of Triphenyltin in a Marine Food Web of Bohai Bay, North China: Comparison to Tributyltin. Environmental Science and Technology 40 (10): 3142 - 3147.

Huet M, Paulet YM, Clavier J (2004). Imposex in *Nucella lapillus*: a ten year survey in NW Brittany. Marine Ecology Progress Series 270: 153 - 161.

Hughes RN, Taylor MJ (1997). Genotype environment interaction expressed in the foraging behaviour of dogwhelks, *Nucella lapillus* (L.), under simulated environmental hazard. Proceedings of the Royal Society of Biological Sciences 264: 417 - 422.

IMO (1999) - [cited 1/07/05]. Harmful effects of the use of anti-fouling paints for ships [on line]. International Maritime Organization, London. Available from: www.imo.org

Janer G, Sternberg RM, Leblanc GA, Porte C (2005). Testosterone conjugating activities in invertebrates: Are they targets for endocrine disruptors? Aquatic Toxicology 71 (3): 273 - 282.

Jelic-Mrcelic G, Sliskovic M, Antolic B (2006). Biofouling communities on test panels coated with TBT and TBT-free copper based antifouling paints. Biofouling 22 (5): 293 – 302.

Karlsson J, Breitholtz M, Eklund B (2006). A practical ranking system to compare toxicity of anti-fouling paints. Marine Pollution Bulletin 52: 1661 - 1667.

Kirby RR, Bayne BL, Berry RJ (1994). Physiological variation in the dog-whelk *Nucella lapillus,* L. either side of a cline in allozyme and karyotype frequencies. Biological Journal of the Linnean Society 53: 277 - 290.

Langston WJ, Pope ND (1995). Determinants of TBT adsorption and desorption in Estuarine Sediments. Marine Pollution Bulletin 31: 32 - 43.

Matthiessen P, Gibbs PE (1998). Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in molluscs. Environmental Toxicology and Chemistry 17 (1): 37 - 43.

Miller KL, Fernandes TF, Read PA (1999). The recovery of populations of dogwhelks suffering from imposex in the Firth of Forth 1987 – 1997/98. Environmental Pollution 106: 183 - 192.

Minchin D, Bauer B, Oehlmann J, Schulte-Oehlmann U, Duggan CB (1996). Biological indicators used to map organotin contamination from a fishing port, Killybegs, Ireland. Marine Pollution Bulletin 34 (4): 235 - 242.

Minchin A, Davies IM (1999). Imposex measurement in the dogwhelk *Nucella lapillus* (L.) – temporal aspects of specimen preparation. Journal of Environmental Monitoring 1: 239 - 241.

Minchin D, Oehlmann J, Duggan CB, Stroben E, Keatinge M (1995). Marine TBT antifouling contamination in Ireland, following legislation in 1987. Marine Pollution Bulletin 30 (10): 633 - 639.

MEPC (2001) - cited [1/07/05]. International Convention on the Control of Harmful Antifouling Systems on Ships [on line], International Maritime Organization, London. Available from: http://www.imo.org/Conventions/mainframe.asp?topic_id=258&doc_id=1494

de Mora SJ (1996). The tributyltin debate: ocean transportation versus seafood harvesting. In de Mora SJ (ed) Tributyltin: case study of an environmental contaminant. No.8, Cambridge environmental chemistry series, Cambridge University Press, Cambridge, pp 1 – 13.

Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T (2004). Involvement of the Retinoid X Receptor in the development of imposex caused by organotins in gastropods. Environmental Science and Technology 28: 6271 - 6276.

Nishikawa J, (2006). Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor. Marine Biology 149: 117 - 124.

Oberdorster E, McClellan-Green P (2000). The neuropeptide APGWamide induces imposex in the mud snail *Ilyanassa obsoleta*. Peptides 21: 1323 - 1330.

Oehlmann J, Bauer B, Minchin D, Schulte-Oehlmann U, Fioroni P, Markert B (1998). Imposex in *Nucella lapillus* and intersex in *Littorina littorea*: interspecific comparison of two TBT-induced effects and their geographical uniformity. Hydrobiologia 378: 199 - 213.

Oehlmann J, Benedetto P, Tillmann M, Duft M, Oetken M, Schulte-Oehlmann U (2007). Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology 16: 29 - 43.

Ohji M, Arai T, Midorikawa S, Harino H, Masuda R, Miyazaki N (2007). Distribution and Fate of Organotin Compounds in Japanese Coastal Waters. Water Air Soil Pollution 178: 255 - 263.

Omae I (2003). Organotin antifouling paints and their alternatives. Environmental Biology and Toxicology 17: 81 - 105.

OSPAR (1998). Joint Assessment and Monitoring Programme (JAMP), Guidelines for contaminant specific biological effects monitoring, Ref. Nº 1998 – 3.

Power AJ, Keegan BF (2001). The significance of Imposex Levels and TBT Contamination in the Red Whelk, *Neptunea antique* (L.) from the Offshore Irish Sea. Marine Pollution Bulletin 42 (9): 761 - 772.

Proud SV (1994). Tributyltin pollution and the bioindicator *Nucella lapillus*: population recovery and community level responses. PhD thesis, University of Liverpool, UK.

Qi S, Zhang S, Qian P, Xiao Z, Li M (2006). Ten new antifouling briarane diterpenoids from the South China Sea gorgorian *Junceella juncea*. Tetrahedron 62: 9123 - 9130.

Rato M, Sousa A, Quintã R, Langston W, Barroso CM (2006). Assessment of inshore/offshore tributyltin pollution gradients in the northwest Portugal continental shelf using *Nassarius reticulatus* as a bioindicator. Environmental Toxicology and Chemistry 25 (12): 3213 - 3220.

Readman JW (2006). Development, Occurrence and Regulation of Antifouling Paint Biocides: Historical Review and Future Trends. Handbook of Environmental Chemistry 5: 1 - 15.

Ronis MJJ, Mason AZ (1996). The metabolism of testosterone by the periwinkle (*Littorina littorea*) in vitro and in vivo: effects of tributyltin. Marine Environmental Research 42: 161 - 166.

Ruiz J, Quintela M, Barreiro R (1998). Ubiquitous imposex and organotin bioaccumulation in gastropods *Nucella lapillus* (L.) from Galicia (NW Spain): A possible effect of nearshore shipping. Marine Ecology Progress Series 164: 237 - 244.

Santos MM, Castro LFC, Vieira MN, Micael J, Morabito R, Massanisso P, Reis-Henriques MA (2005). New insights into the mechanism of imposex induction in the dogwhelk *Nucella lapillus*. Comparative Biochemistry and Physiology Part C 141: 101 - 109.

Santos MM, Reis-Henriques MA, Vieira N, Solé M (2006). Triphenyltin and tributyltin, single and in combination, promote imposex in the gastropod Bolinus brandaris. Ecotoxicology and Environmental Safety 64: 155 - 162.

Santos MM, Ten Haller-Tjabbes CC, Santos AM, Vieira N (2002). Imposex in *Nucella lapillus*, a bioindicator for TBT contamination: re-survey along the Portuguese coast to monitor the effectiveness of EU regulation. Journal of Sea Research 48 (3): 217 - 223.

Santos MM, Vieira N, Santos AM (2000). Imposex in the Dogwhelk *Nucella lapillus* (L.) along the Portuguese Coast. Marine Pollution Bulletin 40(7): 643 - 646.

Smith BS (1971). Sexuality in the American mud snail *Nassarius obsoletus* (Say). Proceedings of the Malacological Society of London 39: 377 - 378.

Smith BS (1981). Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus = Ilyanassa obsoleta.* Journal of Applied Toxicology 1: 141 - 144.

Spooner N, Gibbs PE, Bryan GW, Goad LJ (1991). The effect of tributyltin upon steroids titres in the female dogwhelk *Nucella lapillus*, and the development of imposex. Marine Environmental Research 32: 37 - 49.

Srinivasan M, Swain GW (2007). Managing the use of copper-based antifouling paints. Environmental Management 39: 423 - 441.

Stewart C, de Mora SJ (1992). Elevated tri(n-butyl)tin concentrations in shellfish and sediments from Suva Harbour, Fiji. Applied Organometallic Chemistry 6: 507 - 512.

Takahashi S, Tanabe S, Takeuchi I, Miyazaki N (1999). Distribution and Specific Bioaccumulation of Butyltin Compounds in a Marine Ecosystem. Archives of Environmental Contamination and Toxicology 37: 50 - 61.

Ten Haller-Tjabbes, Wegener J W, Hattum BV, Kemp JF, Hallers ET, Reitsema, TJ, Boon, JP (2003). Imposex and organotin concentrations in *Buccinum undatum* and *Neptunea antiqua* from the North Sea: relationship to shipping density and hydrographical conditions. Marine Environmental Research 55: 203 - 233.

Thomas KV, McHugh M, Waldock M (2002). Antifouling paint booster biocides in UK coastal waters: inputs occurrence and environmental fate. The Science of the Total Environment 293: 117 - 127.

Tillman M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J (2001). Effects of Endocrine Disruptors on Prosobranch Snails (Mollusca: Gastropoda) in the Laboratory. Part III: Cyproterone Acetate and Vinclozolin as Antiandrogens. Ecotoxicology 10: 373 - 388.

Tyler–Walters H (2003) - [cited 25/06/05]. *Nucella lapillus* – Dog whelk. Marine Life information Network: Biology and Sensitivity Key Information Sub–programme [on-line]. Marine Biological Association of the United Kingdom, Plymouth, UK. Available from: http://www.marlin.ac.uk

Waterman BT, Daehne B, Sievers S, Dannenberg R, Overbeke JC, Klijnstra JW, Heemken O (2005). Bioassays and selected chemical analysis of biocide-free antifouling coatings. Chemosphere 60: 1530 - 1541.

Whalen MM, Hariharan S, Loganathan BJ (2000). Phenyltin Inhibition of the Cytotoxic Function of Human Natural Killer Cells. Environmental Research 84 (2): 162 - 169.

WHO (2002). World Health Organization. IPCS Global assessment of the state-of-the-science of endocrine disruptors. Damstra T, Barlow S, Bergman A, Kavlock R, Van der Kraak G (eds) WHO/PCS/EDC/02.2, pp 35 - 50.

Yebra DM, Kiil S, Dam-Johansen K (2004). Antifouling technology – past, present and future steps towards efficient and environmentally friendly antifouling coatings. Progress in Organic Coatings 50: 75 - 104.

Yebra DM, Kiil S, Weinell CE, Dam-Johansen K (2006). Presence and effects of marine microbial biofilms on biocide – based antifouling paints. Biofouling 22 (1): 33 - 41.

Chapter 2 – Imposex induction by TBT in *Nucella lapillus* (L.)

2.1 – Introduction

Imposex is regarded as a very specific biological response to TBT and TPT environmental contamination and for this reason it has been used worldwide as a most useful tool to monitor the levels of TBT and TPT pollution. However, in the case of species - like *Nucella lapillus* - that may present strong genetic and phenotypic geographical variation, it is important to assess if the imposex response may also vary geographically due to different sensitivities of the populations to this type of pollution. For instance, Gibbs (1993) described the possible occurrence of the Dumpton Syndrome in *N. lapillus*, which is a rare genetic deficiency that lessens the imposex degree and the sterilization in females at highly polluted sites. However, excluding the case of Dumpton Syndrome, geographical uniformity of the imposex response was already addressed by Gibbs *et al.* (1991) by showing that dogwhelks from four different sites around the British Islands developed comparable imposex intensities and accumulated identical amounts of TBT after being exposed to the same TBT concentrations at the laboratory. Also Oehlmann and co-workers (1998) demonstrated that this species showed the same concentration-effect relationship over an even more extended geographical range by the analysis of specimens found in Ireland, France and Germany. Although, there is a good evidence that there is a geographical uniformity of the imposex response in dogwhelks throughout Europe, we found that in Ria de Aveiro (NW Portugal) there was a site (Forte da Barra) where dogwhelks were displaying lower levels of imposex than we could expect, considering the close proximity of a commercial port and the relatively high levels of TBT in their tissues. In the current study we aim to test, under laboratory conditions, if dogwhelks collected from Forte da Barra may show a different imposex response comparing to identical animals of a nearby population that presented a more typical relationship between imposex and TBT tissue contamination.

2.2 – Material and Methods

Dogwhelks were collected in June of 2007 from two sites in Ria de Aveiro: Forte da Barra and Marégrafo. These sites were selected because of the different apparent sensitivity of its dogwhelks population to TBT pollution. Forte da Barra (41º38'56N - 8º43'59W) is located near the North Commercial terminal of the Aveiro Port whereas Marégrafo (40º38'71N – 8º44'82W) is located at the entrance of Ria de Aveiro, just 1.3 Km apart from the former sampling station.

2.2.1 – Sampling and experiment preparation

The dogwhelks' collection was performed in two different days, since a low tide is required to assemble the needed animals. Approximately 400 animals were collected from Forte da Barra and 300 from Marégrafo.

At the lab, dogwhelks were narcotized with a solution of MgCl at 7% in distilled water for about 40 minutes. Subsequently, all females were analysed under a stereo microscope and both penis length (FPL) and vas deferens sequence (VDS) were assessed. Females with FPL between 0.28 and 1.40 mm were selected from each sampling site. Exposure was performed in 1L glass bottles containing 5 females and each glass bottle constitutes a replicate (Figure 8). The females of Forte da Barra and of Marégrafo were separately exposed to a nominal TBT concentration of 100ng TBT-Sn/L during 45 days. As Dimethyl sulfoxide (DMSO) was used as a solvent for the TBT solution, a control group of females were exposed to DMSO (20µL per litre of seawater) for the same period of time. A seawater control was not used as it is reported that DMSO does not induce imposex in neogastropod species like *N. lapillus* (Santos *et al.*, 2005) and *N. reticulatus* (Barroso *et al.*, 2002; Sousa *et al.*, 2005).

A total of 5 replicates were used in the case of Forte da Barra but only 3 for Marégrafo due to the low number of females found at this site with the required imposex level. The replicates of five dogwhelks were strategically made so that each animal could be followed individually throughout the experiment. Photographs of every replicate were also taken to assure that each animal could be always identified.

On the following day, dead or low condition animals were eliminated and substituted. Solutions were constantly aerated and renewed once a week. Salinity was approximately 35 psu and water temperature was 18.5 ± 0.5ºC. All material used was glass-made and were previously washed with tap water and soap, followed by a 3 times washing with acetone and 1 time washing with hexane in order to prevent any glass material contamination. After this cleaning procedure the bottles were washed with seawater.

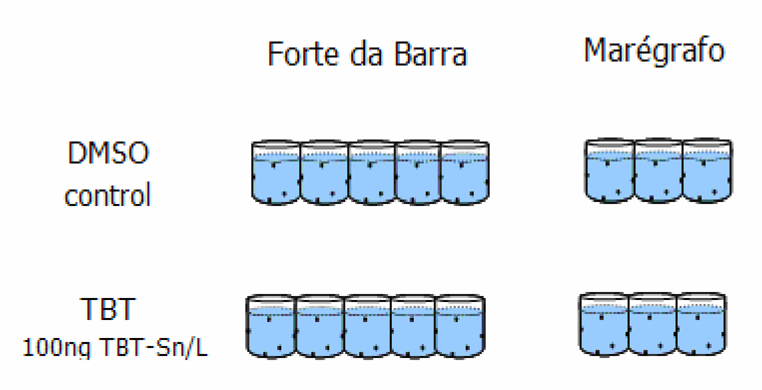


Figure 8 – *Nucella lapillus.* Experimental design used for assessing the development of imposex in dogwhelks collected from two different sites in Ria de Aveiro: Forte da Barra and Marégrafo.

2.2.2 – Imposex indices assessment

The Female Penis Length (FPL) was measured (see point 1.6.2) with a stereo microscope using a graduated eyepiece to the nearest 0.01 millimetres. The development of the vas deferens was classified using the Vas Deferens Sequence (VDS) proposed by

Gibbs and Bryan (1987) already described in Chapter 1 (point 1.6.2). At the beginning of this experiment, some VDS stages were hard to assess once it was not possible the cut and pin back of the mantle. Dogwhelks' body could only be seen until a certain limit by pushing it out of the shell after narcotisation. This fact imposed some limitations with the differentiation between stage 3 and 4 since the vas deferens could unite in an area where we could not see it (nearer to the genital papilla).

2.2.3 – Test Solutions

Seawater was prepared at the lab with sea salt (Crystal Sea, marine mix – marine enterprise international) and filtered and sterilized tap water, which was aerated during 2 days before adding the salt. TBT solutions were prepared with TBT chloride ($C_{12}H_{27}ClSn$, Fluka) and the solvent DMSO ($(CH_3)_2SO$, MERCK); 20µL of this solution were added to each litre of artificial seawater).

2.2.4 – Statistical analysis

The non parametric Wilcoxon matched pairs test ("W test") was used to assess differences on FPLI and VDSI between the measurements effectuated in each animal, at the beginning and at the end of the experiments. The Mann-Whitney U test ("U test") was used to assess differences on FPLI and VDSI between two independent groups of animals (control and the TBT treatment), at the same moment of the exposure time.

Both tests can provide different ways of analysing the data. The W test is a very powerful test since it compares individual subjects in a sample before and after a given treatment. The U test provides a more general analysis of differences between two independent samples. We provide these two alternative methods in order to explore their behaviour and get a more sensible analysis of the results. All statistical data analysis were performed in Statistica 6.0 software.

2.3 – Results and Discussion

In general, *N. lapillus* females from Forte da Barra and Marégrafo presented higher levels of imposex after 45 days of exposure to 100ng TBT-Sn/L than in the beginning of the experiment (Table 2, Figure 9 and Figure 10). The females of Forte da Barra that were exposed to TBT showed a significant increase of the penis (Wilcoxon test, $W=3.6$, $p<0.01$) from the beginning of the experiment (mean value of 0.86mm) to the end of the experiment (1.25mm); similarly, the VDSI in these females increased significantly ($W=3.3$, $p<0.01$) from a mean value of 2.36 to 2.95. In the control group from this sampling station there was no significant difference between both parameters after 45 days ($W=1.5$, $p=0.2$). Hence, using the W test, it is clear that TBT caused imposex development in the dogwhelks. This test is very useful because it takes into account the information regarding the variability of the individuals and equilibrates possible differences in the initial imposex condition in the control and the TBT treatments. It is also possible to use the Mann-Whitney U test to compare the following differences: between the mean value of imposex in the two groups of females at day 0 (control and TBT treatments) and between those same groups after the 45 days of exposure. This test shows significant differences, in the FPLI ($U=32.5$, $p<0.001$) and VDSI ($U=90.0$, $p<0.05$), between females in the control after 45 days and the ones exposed to TBT during the same period. At the beginning of the exposure (day 0), both control and TBT groups were compared and no significant differences were found between them ($U=111.0$, $p=0.97$ for FPLI and $U=103.5$, $p=0.71$ for VDSI) (Figure 9).

The females of Marégrafo showed a significant increase of the penis after 45 days in the control, from 0.80 to 0.95mm ($W=2.0$, $p<0.05$) and in the TBT treatment, from 0.85 to 1.67mm ($W=2.6$, $p<0.01$). The FPL increase in the control group was not expected and deserves further research in the future. The VDSI in these females showed a significant increase from 2.37 to 2.96 ($W=2.0$, $p<0.05$) only in the TBT treatment, which indicates a positive imposex induction by this compound. The U test also indicates that TBT caused a significant increase of the penis length on exposed females ($U=36.5$, $p<0.05$). The difference in the VDSI, in this case, is not significant ($U=29.5$, $p=0.31$), which may indicate that this parameter is less sensitive than FPL for short-term imposex induction (Figure 10).

The results confirm that TBT does induce imposex in *N. lapillus*, though in one case this induction failed to be significant, most probably due to the short-term of the experiment, to the low number of animals (and replicates) used and some mortality that occurred during the experiment (0-40% across replicates). It is important to note that there was a concern to avoid collecting a high number of animals from the sampling sites, considering that this species is not very abundant inside Ria de Aveiro.

Table 2 – *Nucella lapillus*. Number of animals that survived in each replicate for assessing the development of imposex in dogwhelks.

<table>
<tr><th>Series</th><th>Treatments</th><th>Replicates</th><th>N</th></tr>
<tr><td rowspan="6">Marégrafo</td><td rowspan="3">control</td><td>R1M</td><td>5</td></tr>
<tr><td>R2M</td><td>4</td></tr>
<tr><td>R3M</td><td>5</td></tr>
<tr><td rowspan="3">100 ng TBT-Sn/L</td><td>R4M</td><td>3</td></tr>
<tr><td>R5M</td><td>3</td></tr>
<tr><td>R6M</td><td>4</td></tr>
<tr><td rowspan="9">Forte da Barra</td><td rowspan="4">control</td><td>R1F</td><td>4</td></tr>
<tr><td>R2F</td><td>4</td></tr>
<tr><td>R3F</td><td>4</td></tr>
<tr><td>R4F</td><td>3</td></tr>
<tr><td rowspan="5">100 ng TBT-Sn/L</td><td>R9F</td><td>4</td></tr>
<tr><td>R10F</td><td>3</td></tr>
<tr><td>R11F</td><td>4</td></tr>
<tr><td>R12F</td><td>4</td></tr>
<tr><td>R13F</td><td>5</td></tr>
</table>

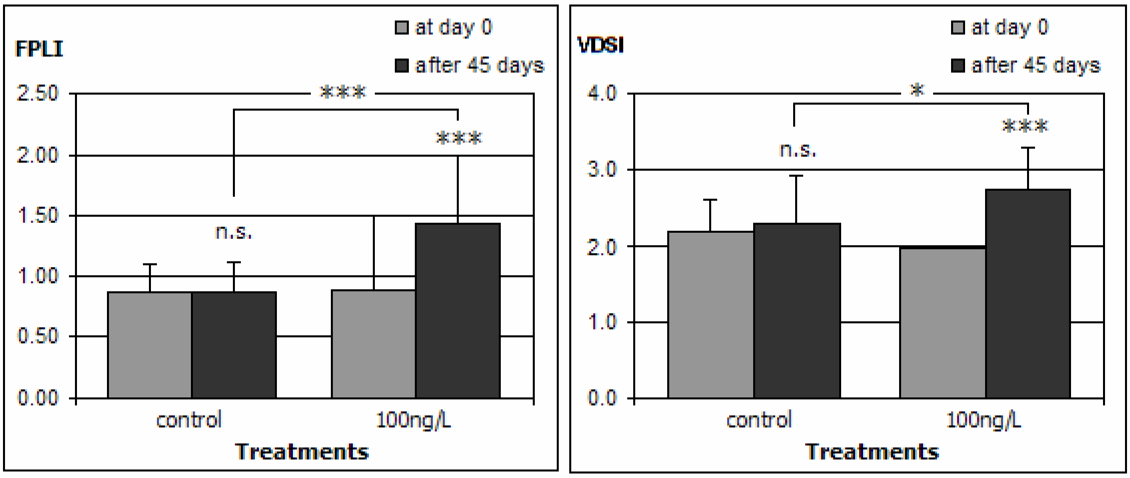


Figure 9 – *Nucella lapillus*. Female Penis Length Index (FPLI) and Vas Deferens Sequence Index (VDSI) development after 45 days of exposure to 100ng TBT-Sn/L in dogwhelks collected from Forte da Barra.

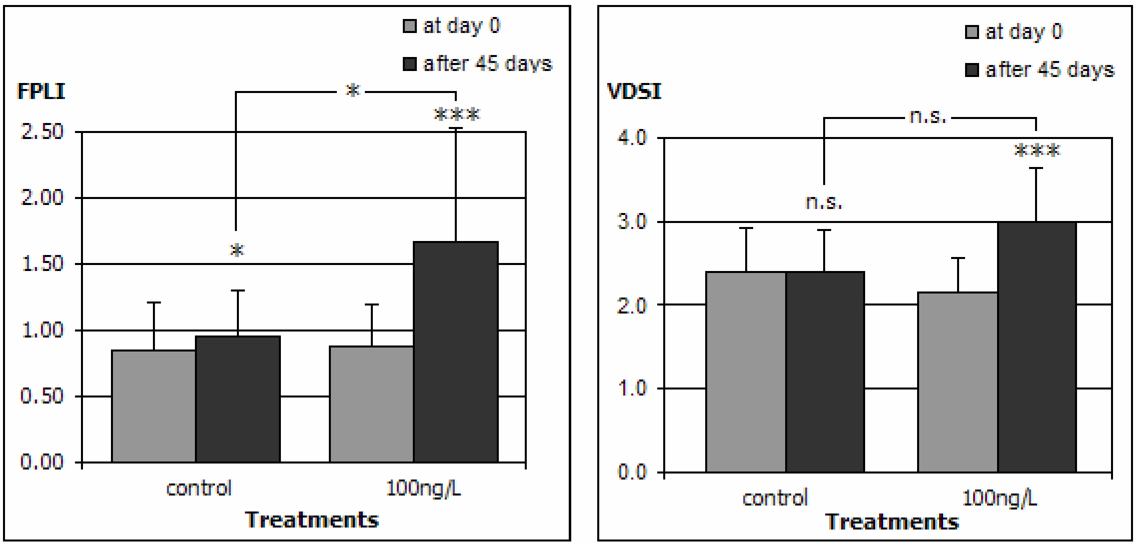


Figure 10 – *Nucella lapillus*. Female Penis Length Index (FPLI) and Vas Deferens Sequence Index (VDSI) development after 45 days of exposure to 100ng TBT-Sn/L in dogwhelks collected from Marégrafo.

When we compare the imposex development in females collected from Forte da Barra and the ones sampled in Marégrafo, after 45 days of exposure, we find that there is no significant difference in the FPLI and VDSI between controls from both sites (U test=93.0, p=0.60 and U test=57.0, p=0.87 respectively) and between the TBT exposed females of Forte da Barra and Marégrafo (U test=98.5, p=0.95 and U test=47.5, p=0.46, respectively) (Figure 11). At the beginning of the experiment, significant differences were also not found between control and TBT treatments. These results show that there is no apparent different sensitivity of both populations to TBT contamination, at least for a period of time used in this experiment.

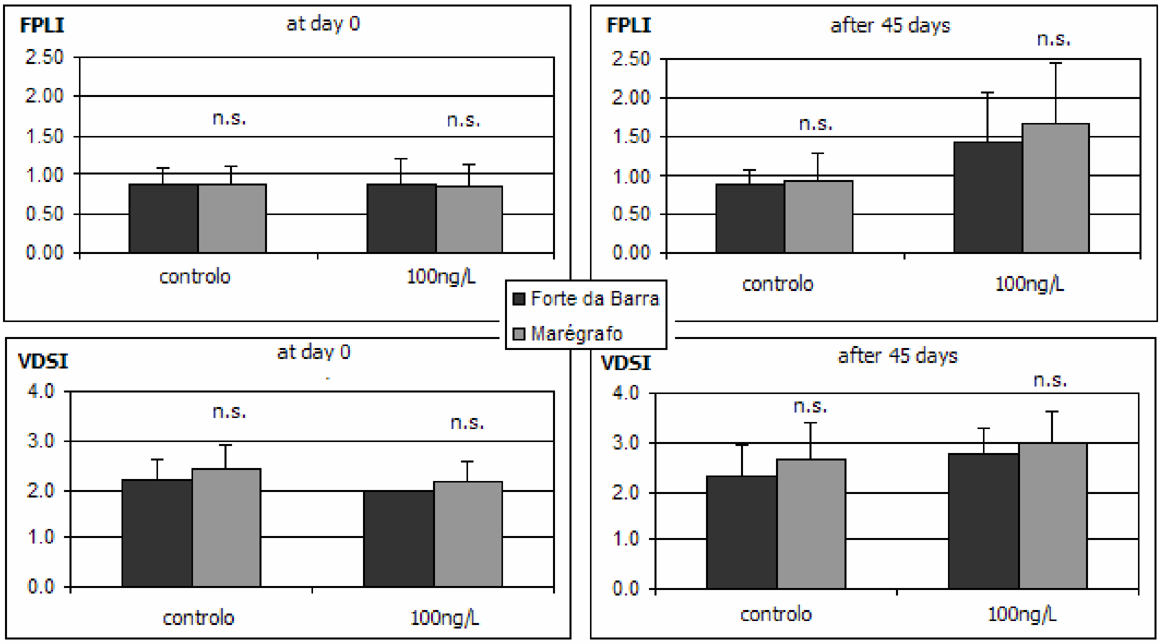


Figure 11 – *Nucella lapillus***.** Female Penis Length Index (FPLI) and Vas Deferens Sequence Index (VDSI) for dogwhelks collected at Forte da Barra and Marégrafo, at the beginning and at the end of the experiment.

2.4 – Conclusion

In general, the results obtained show that TBT induces the development of imposex in *N. lapillus*. Many authors had already proved that, when dogwhelk females are exposed to TBT, masculinisation proceeds in a dose-responsive manner (Bryan *et al.*, 1988; Bryan and Gibbs, 1991; Tillman *et al.*, 2001, Santos *et al.*, 2005). Hence, the results described in this chapter only upholds the well-established concept that imposex is caused by TBT. However, the main objective was to investigate if there was any kind of differential response to TBT by different populations of dogwhelks. A recent survey performed in 2006 in the Ria de Aveiro (unpublished data) showed that *N. lapillus* from Forte da Barra displayed a VDSI of 1.8 and a RPSI of 9.7% whereas female tissues presented 126ng TBT-Sn/g dw. These imposex values were in fact much lower than other surrounding populations like, for instance, Marégrafo where VDSI was 3.0 and RPSI was 41.4% for a TBT contamination of 10ng TBT-Sn/g dw in female's tissues. Considering that Forte da Barra presented, in 1998, the highest levels of imposex for this species in the Ria de Aveiro (with female sterilization) (Barroso *et al.*, 2000), there was a concern that perhaps they could have developed some kind of resistance to TBT pollution. The experiment performed in the current chapter, in order to investigate this hypothesis, constitutes a very quick and simple approach but, anyway, these results indicate that such a resistance phenomenon is not clearly evident. However, further laboratorial experiments should be carried on in the future using longer periods of exposure and a larger number of replicates.

Our results corroborate previous studies suggesting that there is a considerable geographical uniformity in the imposex response of *N. lapillus* populations to TBT pollution, which validates the use of this species for monitoring purposes.

2.5 – References

Barroso CM, Moreira MH, Gibbs PE (2000). Comparison of imposex and intersex development in four prosobranch species for TBT monitoring of a southern European estuarine system (Ria de Aveiro, NW Portugal). Marine Ecology Progress Series 201: 221 - 232.

Barroso CM, Reis-Henriques MA, Ferreira MS, Moreira MH (2002). The effectiveness of some compounds derived from antifouling paints in promoting imposex in *Nassarius reticulatus*. Journal of the Marine Biological Association of the United Kingdom 82: 249 - 233.

Bryan GW, Gibbs PE, Burt GR (1988). Comparison of the effectiveness of tri-n-butyltin chloride and five other organotin compounds in promoting the development of imposex in the dog-whelk *Nucella lapillus.* Journal of the Marine Biological Association of the United Kingdom 68: 733 - 744.

Bryan GW, Gibbs PE (1991). Impact of Low Concentrations of Tributyltin (TBT) on Marine Organisms: A Review. In Newman MC, McIntosh AW (eds) Metal Ecotoxicology Concepts and Applications Lewis Publishers, Michigan, pp 323 - 361.

Gibbs PE (1993). A male genital defect in the dog-whelk, *Nucella lapillus* (neogastropoda), favouring survival in a TBT-polluted area. Journal of the Marine Biological Association of the United Kingdom 73: 667 - 678.

Gibbs PE, Bryan GW, Pascoe PL (1991). TBT-induced imposex in the dogwhelk, *Nucella lapillus*: geographical uniformity of the response and effects. Marine Environmental Research 32: 79 - 87.

Oehlmann J, Bauer B, Minchin D, Schulte-Oehlmann U, Fioroni P, Markert B (1998). Imposex in *Nucella lapillu*s and intersex in *Littorina littorea*: interspecific comparison of two TBT-induced effects and their geographical uniformity. Hydrobiologia 378: 199 - 213.

Santos MM, Castro LFC, Viera MN, Micael J, Morabito R, Massanisso P, Reis-Henriques MA (2005). New insights into the mechanism of imposex induction in the dogwhelk *Nucella lapillus*. Comparative Biochemistry and Physiology Part C 141: 101 - 109.

Sousa A, Génio L, Mendo S, Barroso C (2005). Comparison of the acute toxicity of tributyltin and copper to veliger larvae of *Nassarius reticulatus* (L.). Applied Organometallic Chemistry 19: 324 - 328.

Tillman M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J (2001). Effects of Endocrine Disruptors on Prosobranch Snails (Mollusca: Gastropoda) in the Laboratory. Part III: Cyproterone Acetate and Vinclozolin as Antiandrogens. Ecotoxicology 10: 373 - 388.

Chapter 3 - Imposex levels around North Wales: spatial and temporal evolution

3.1 – Introduction

The negative impacts of the tributyltin (TBT) pollution on the aquatic ecosystems lead the application of legislative restrictions on the use of TBT antifouling paints in the UK coastal waters. The first regulation came into effect on January 1986 with the restriction of the total tin content in dried co-polymer and free-association paints to a maximum of 7.5 and 2.5%, respectively. It also lead to the creation of guidelines for the AF coating's painting and removal, the implementation of research and monitoring programs and the creation of a first Environmental Quality Target (EQT) of 8ng Sn/L in water (Waldock *et al.*, 1987). As TBT in coastal areas frequently exceeded this EQT, the UK government considered the application of further restrictions on the retail sale of TBT AF systems (Abel *et al.*, 1987). In 1987, legislation came into force announcing a prohibition on a large scale and on supply of TBT AF paints and its application on nets and fishing cages used in fishing farms and, above all, in pleasure crafts less than 25m in length. The EQT for water-borne TBT was lowered to 0.8ng Sn/L and replaced by an Environmental Quality Standard (EQS) (Cleary, 1991). The IMO 2001 convention, forbidding the application or re-application of organotin compounds on ships from January 2003, was put in place in EU legislation through the Directive 2002/62/EC, which prohibits marketing and use of tin organic compounds intended to be used as AF agents or AF products on all kinds of vessels of all length. The EC Regulation 782/2003 forbided the use of OT based AF paints from the 1st of July 2003 (www.imo.org).

Thus, it is important to perform a regular surveillance on the degree of TBT pollution in coastal waters in order to evaluate the effectiveness of the above legislation.

The Oslo and Paris Commission (OSPAR) adopted the imposex response as a component of their international Joint Assessment and Monitoring Programme (JAMP) and recommended the dogwhelk *N. lapillus* (L.) as a sensitive bioindicator of TBT pollution (OSPAR, 1998). The present work aims to assess the levels of imposex and organotin contamination in this species around the North Wales in 2006 and compare them with similar data reported by different authors for the past 20 years, in order to evaluate the evolution of TBT pollution and assess the efficacy of the legislation in this area.

3.2 – Material and methods

Dogwhelks were sampled from 20 different sites (Figure 12) around Anglesey and the Lleyn Peninsula, North Wales, between October and December of 2006, in order to assess imposex levels and population abundance. These sites were the same previously sampled by Meredith (1987), Dukes (1994), Wagiman (1995), Hughes (1997), Saurel (2002), Chatzinikolaou (2004) and our team in 2004/2005. We selected the same locations in order to allow temporal comparisons of imposex levels so that we can understand the evolution of TBT pollution in North Wales' coast. Besides, the sites selected in the current survey allow a wide geographical coverage of this coastal area and are subjected to different intensities of boat traffic, which may give a good idea of how this factor can influence the imposex levels.

The current work provides in Annex I a description of each sampled site regarding its location, physiography, dominant organisms' coverage, hydrodynamism and local human activities, with particular relevance to boat traffic – ports, marinas, mooring areas, dry docks, etc. It is also provided a histogram for each site with the number and size of dogwhelks caught for the timed searching period (see point 3.2.1). The characterization here provided can be useful for future surveys as this work aims also to create a baseline for long term imposex monitoring studies along North Wales' coast.

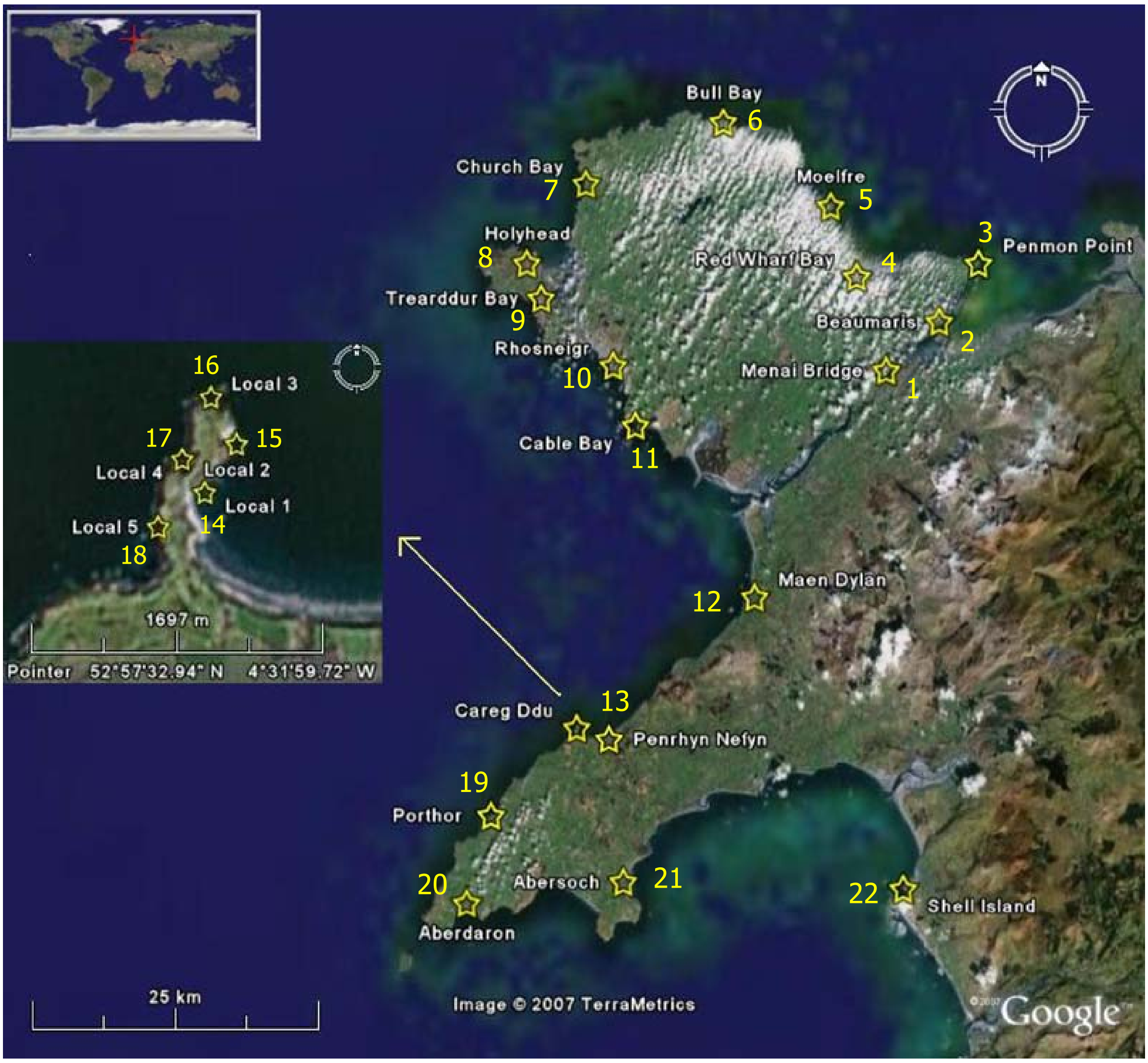


Figure 12 – *Nucella lapillus***.** Sampling sites around the coast of Anglesey and Lleyn Peninsula, North Wales (Google maps, 2007).

3.2.1 – Sampling

At every site, with the exception of Porthor (due to bad weather conditions), animals were collected using three timed searches, of 5 minutes each, in order to access dogwhelks abundance along the different shores. This type of collection gives an approximate idea of the abundance and size frequency distribution of the populations at each study site. A more reliable method - the quadrat sampling - was employed previously but it consumed too much time that does not allow a short term coast survey - a requirement for imposex monitoring - and for this reason it was abandoned.

All collected animals were placed in labelled buckets, filled with seawater, fitted with lids and brought back to the laboratory. Once at the laboratory, each bucket was placed on a circulation bench with a supply on running and filtered natural seawater. Animals were processed in a maximum period of 4 days. During this time mortality was negligible.

3.2.2 – Imposex measurement

Whenever possible, a minimum of 20 adult males and 20 adult females were observed in order to obtain comparable and significant results. The presence of a thick shell lip in combination with a high shell height (>15mm) were used to recognize the adults. After shell height (maximum distance between the apex and the base of the shiphonal channel of the shell) measurement with a vernier calliper to the nearest 0.05mm was made, each animal was cracked open using a bench vice. Pressure had to be applied between the dorsal part of the shell and the aperture face in order to create a clean fracture. Each individual was then removed undamaged, once the columella muscle of the animal was detached from the shell with the help of a pair of forceps. The body of the dogwhelk was then placed dorsal side up in a Petri dish filled with seawater. The dish was placed under a dissecting microscope (Leica wild M3Z) and illuminated with a cold light source (Fibre optic illuminator KL 1500 electronic Schott) so that the penis tissue would not be damaged. The sex was determined for each dogwhelk as described in

Chapter 1 point 1.6.1. The Female Penis Length Index (FPLI), the Relative Penis Size index (RPSI), the Vas Deferens Sequence Index (VDSI) and the percentage of females affected by imposex (%I) were determined for each station (see section 1.6.2). Parasitized specimens were discarded from the analysis.

3.2.3 – Organotin content in the tissues

OTs analysis was performed following the method described by Sousa *et al.* (2007) with some modifications. Briefly, about 1g of freeze dried sample was spiked with 50ng of internal standards including deuterated butyltins (d_9-MBT, d_{18}-DBT and d_{27}-TBT), phenyltins (d_5-MPT, d_{10}-DPT and d_{15}-TPT), and octyltins (d_{17}-MOT, d_{34}-DOT and d_{51}-TOT) and homogenized with 1N HBr/methanol-ethyl acetate (1:1) solution using a Polytron homogenizer. The homogenate was centrifuged (15 min at 3000 rpm), and the supernatant was transferred to decantation balloons with 50ml of NaBr saturated water and 15ml of ethylacetate/hexane (3:2). After extraction (by shaking for 10 min, twice) 100ml of hexane was added into the extract and water phase was discarded. Then the organic layer was dehydrated with anhydrous sodium sulphate and concentrated (using a rotary evaporator) near to dryness. The concentrate was solved into small amount of ethanol and mixed with 5ml of 1M acetate buffer (pH 5.0). Organotins in the extract were then ethylated by adding 1ml of 5% tetraethyl sodium borate. After ethylation (by shaking for 15 min) 40ml of 2M KOH was added to the mixture that was shaken one hour to decompose the fat. After saponification, the ethylated organotins were re-extracted by hexane. Afterwards the extract was dehydrated by sodium sulphate, concentrated by a rotary evaporator and cleaned up using a SEP-PAK plus florisil cartridge. The final solution was concentrated under a gentle nitrogen flux to 1ml and spiked with 50ng of deuterated tetrabutyltin as a recovery standard. The final solutions were then injected into a gas chromatograph.

The quantification of OTs was conducted by a gas chromatograph equipped with a mass spectrometer (GC-MS) (Hewlett-Packard 6870 GC system with 5973 mass selective detector and 7683 series auto sampler). GC-MS was equipped with a fused silica capillary column (0.25 mm i.d. x 30m length consisted of DB-1: 100% dimethylpolysiloxane, 0.25μm bounded phase) and operated in electron impact and selected ion monitoring mode (EI-SIM). The concentrations of OTs were calculated based on the peak areas of target compounds and their deuterated surrogates as internal standards following an internal standard isotope dilution method. Calibration curves for monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), monophenyltin (MPT), diphenyltin (DPT), triphenyltin (TPT), monoctyltin (MOT), dioctyltin (DOT) and trioctyltin (TOT) were made from the analysis of standard solutions showing 4 levels of native compound concentrations (5, 10, 50 and 250ng/ml) with a constant concentration of internal and recovery standards (50ng/ml). Recoveries of internal standards through the whole analytical procedure were estimated based on the peak areas of internal and recovery standards. MBT, DBT, TBT, MPT, DPT, TPT, MOT, DOT and TOT average recovery rates (±St Dev) were 43.9±6.70, 89.6±4.08, 94.0±3.54, 16.6±6.53, 80.5±8.11, 129.4±13.14, 60.52±10.60, 122.38±15.02 and 157.2±51.80%, respectively.

To assess the QA/QC of measurements in this study, certified reference material of fish tissue (NIES CRM No.11) was analyzed by the method described above. Results obtained from 3 replicate analysis (1.2±0.1μg/g as TBTCl and 6.3±0.1μg/g as TPTCl) are in accordance with the certified/reference values reported (1.3±0.1μg/g as TBTCl and 6.3±0.1μg/g as TPTCl). In addition, a procedural blank was included with each analytical batch to check for interfering compounds and to correct sample values, if necessary. The detection limits of each organotin compound were calculated based on deviation (3σ) of each peak area when the standard solutions containing low levels of native compounds (1 or 5ng/ml) were measured by GC-MS. If any peak was detected in the blank sample, detection limit was determined as quantities of three times those peak areas. In this study, concentrations of OTs were described in terms of ng Sn/g dw. Our methods gave detection limits of: 5.1 for MBT, 0.5 for DBT, 0.1 for TBT, 0.1 for DPT, 0.1 for TPT, 0.2 for DOT and 0.1 for TOT. MPT and MOT were not analyzed.

3.2.4 – Statistical analysis of the data

The Mann-Whitney U test was used to assess the significance of the temporal differences in the imposex levels of *N.lapillus* for each site. This is a non parametric test used to compare two independent groups. It is an alternative to the t-test when the assumption of normality or equality of variance is not met or can not be tested (Sokal and Rolf, 1987).

The non parametric Friedman test and the Wilcoxon matched pairs test were used to compare repeated observations in different years, on the same stations depending whether comparisons were being made between three or two years simultaneously. These tests allow the assessment of global temporal differences in the imposex levels. Friedman´s test was used to make comparisons between 1987, 1994/95 and 2006 in the two main regions of the study area: Anglesey and Lleyn Peninsula; whilst the Wilcoxon test was used to assess global temporal differences between 2004/2005 and 2006 surveys along North Wales' coast.

All statistical data analysis were performed in Statistica 6.0 software.

3.3 – Results and Discussion

3.3.1 – Spatial variation of imposex in *Nucella lapillus*

Imposex indices (FPLI, RPSI, VDSI and %I) of *N. lapillus* for the 20 sites around Anglesey and Lleyn Peninsula, North Wales, in 2006 are presented in Table 3 and Figures 13 and 14.

Table 3 – *Nucella lapillus*. Data relative to the 20 sites around North Wales: Observed animals' number (N), mean shell heights (Shell Height), Relative Penis Size Index (RPSI), Vas Deferens Sequence Index (VDSI), and Female and Male Penis Length Indices (FPLI and MPLI, respectively) of individuals assessed from both genders. Standards deviation of VDSI, FPLI and MPLI are also presented (SD).

Sites		Females						Males				RPSI (%)
Code	Name	N	Shell height (mm)	FPLI (mm)	SD	VDSI	SD	N	Shell height (mm)	MPLI (mm)	SD	
1	Menai Bridge	39	29.40	0.25	0.30	1.6	0.8	33	28.75	4.25	1.20	0.020
2	Beaumaris	36	31.50	1.60	0.80	3.4	0.8	33	31.03	5.45	0.70	2.530
3	Penmon Point	22	27.35	0.31	0.50	1.0	1.2	30	26.97	4.68	1.00	0.030
4	Red Warf Bay	43	29.33	0.45	0.50	1.7	1.0	35	27.13	4.99	1.10	0.075
5	Moelfre	31	29.31	0.08	0.20	0.8	0.7	30	29.08	4.99	1.00	0.000
6	Bull Bay	29	28.83	2.19	1.90	2.1	1.0	32	28.55	4.50	0.40	11.47
7	Church Bay	33	26.68	0.22	0.40	1.0	1.0	30	26.20	4.14	1.24	0.015
8	Hollyhead	25	29.27	0.02	0.10	0.7	0.5	30	29.07	4.70	1.10	0.000
9	Trearddur Bay	36	28.58	0.20	0.40	1.2	0.8	32	28.09	4.38	0.90	0.009
10	Rhosneiger	36	26.31	0.12	0.30	0.9	0.9	25	25.74	4.15	0.70	0.003
11	Cable Bay	34	26.58	0.13	0.20	0.9	0.9	34	26.12	3.95	0.70	0.003
12	Maen Dylan	21	23.84	0.00	0.00	1.0	0.2	11	24.60	4.50	0.80	0.000
13	Penrhyn Nefyn	47	28.43	1.20	0.40	3.2	0.7	32	28.10	4.93	1.04	1.435
14	Careg Ddu – L1	34	28.87	1.78	0.70	3.8	0.5	32	28.76	4.96	0.70	4.624
15	Careg Ddu – L2	37	28.99	0.80	0.70	2.5	1.0	40	28.30	4.76	0.80	0.468
16	Careg Ddu – L3	30	28.24	0.22	0.20	1.5	0.8	33	27.57	4.39	0.80	0.013
18	Careg Ddu – L5	32	28.86	0.05	0.20	0.6	0.6	32	28.56	4.55	0.60	0.000
19	Porthor	32	28.44	0.06	0.20	1.0	0.7	22	28.12	4.81	0.80	0.000
21	Abersoch	31	28.64	0.00	0.00	0.5	0.5	31	28.78	5.03	1.06	0.000
22	Shell Island	46	28.75	0.02	0.10	0.6	0.6	32	28.50	5.13	0.50	0.000

All the 20 sites surveyed exhibited females with imposex. As it can be seen in Figure 13, the highest imposex incidence was observed at Beaumaris (st. 2), Penrhyn

Nefyn (st. 13) and Careg Ddu – L1 and L2 (st.14 and 15) with values of 100%, followed by Menai Bridge (st. 1), Red Warf Bay (st. 4), Bull Bay (st. 6), Maen Dylan (st. 12) and Careg Ddu – L3 (st. 16) that showed %I values above 90%. The lowest %I value (and the only one below 50%) was found at Abersoch (st. 20).

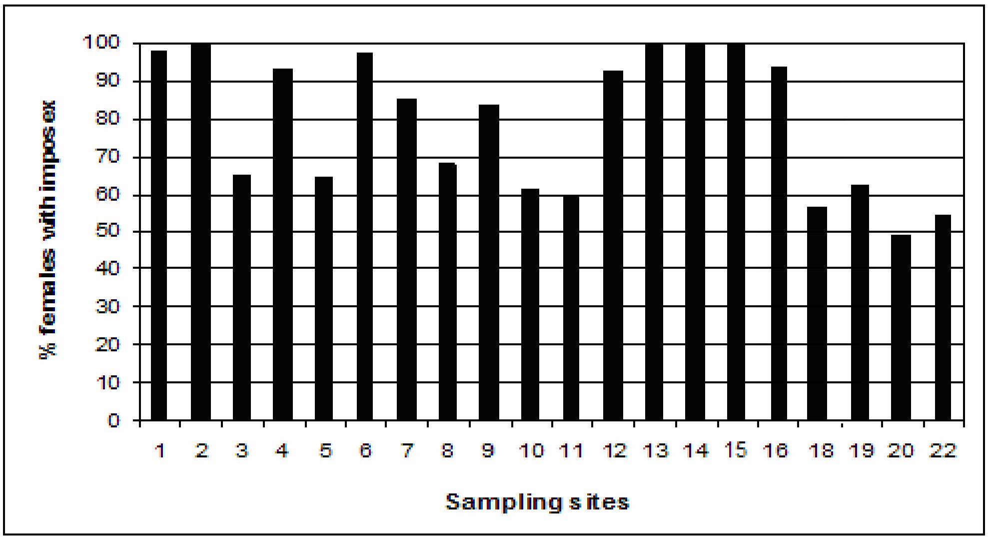


Figure 13 – *Nucella lapillus*. Percentage of affected females (%I) by sampling site.

The highest VDSI values were observed at Careg Ddu – L1 (3.8), Beaumaris (3.4) and Penrhyn Nefyn (3.2) (Table 3 and Figure 14). In the entire study only two sterile females were found, one at Beaumaris and other at Penrhyn Nefyn, but these animals did not carry aborted egg capsules. The lowest VDSI values were found at Abersoch (0.5) followed by a group of other sites: Shell Island (0.6), Careg Ddu – L5 (0.6), Hollyhead (0.7), Moelfre (0.8) and Maen Dylan (1.0). It is interesting to note that VDSI is, in fact, the most meaningful imposex parameter not only because it assesses the reproductive capacity of the population but also because it denotes the start up of virilisation in females; for instance, at Abersoch and Maen Dylan, the VDS individual stages did not surpass 1 and, consequently, the FPLI was 0. The global distribution of mean VDSI values around North Wales can be seen in Figure 15.

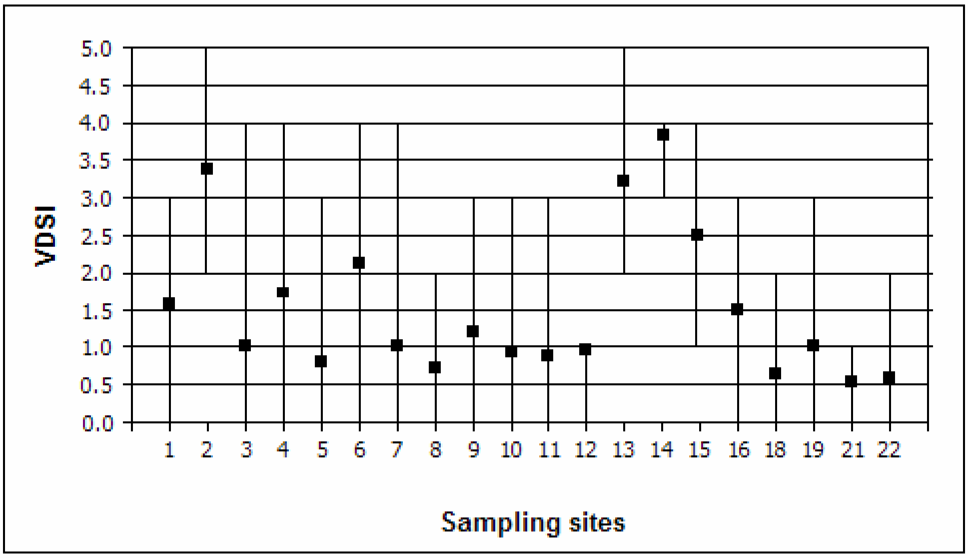


Figure 14 – *Nucella lapillus*. Mean VDSI and its range (maximum and minimum value) by sampling site.

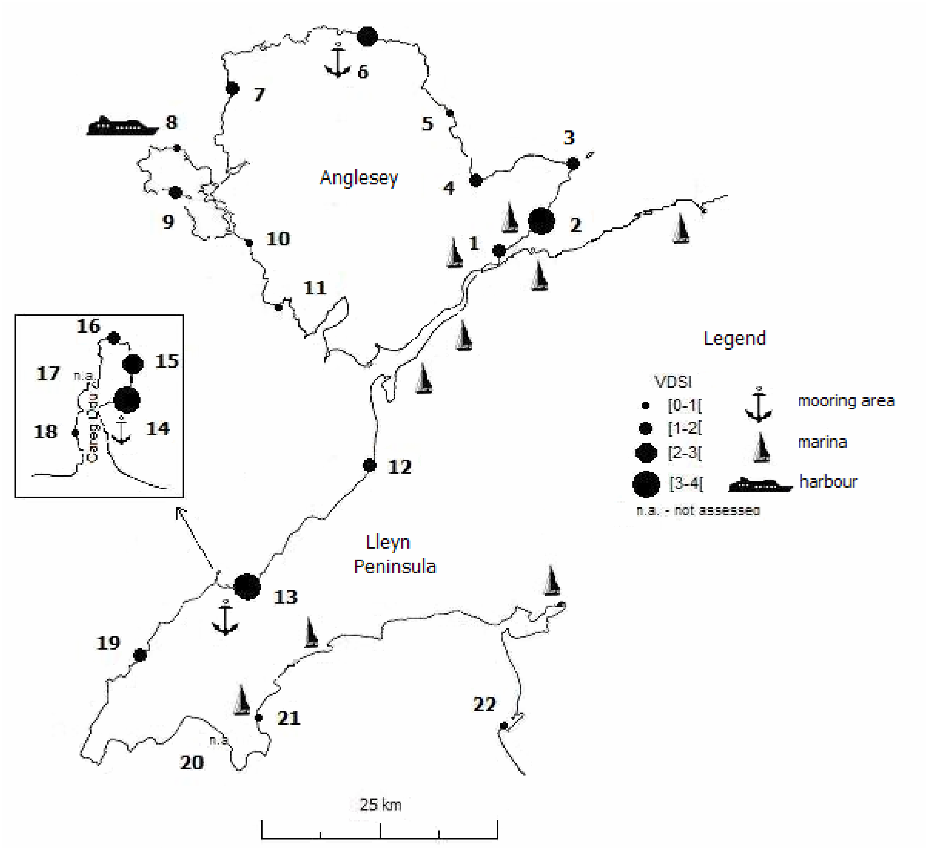


Figure 15 – *Nucella lapillus*. Spatial distribution of Vas Deferens Sequence Index (VDSI) and possible TBT sources in North Wales.

No clear relation can be seen between imposex levels and the proximity of localized boat activities. For instance, Hollyhead harbour was thought to be the major source of TBT in the area but the sites located nearby (sts. 8, 7 and 9) do not present high imposex values. Low values were also found at Abersoch (st. 20) and Shell Island (st. 22), despite the presence of several marinas and the high amount of fishery/leisure aquatic activities in the area. However, some sites can be related with TBT hotspots: Beaumaris' high imposex indices can be somehow related with the presence of several marinas in the Menai Strait. Some of these marinas did the painting and removal of boat AF coatings before the legislative ban prohibiting small boats from having TBT in their hulls. Although this ban took place in 1987, it is known that sediments can trap organotins for many years. Also, in the Careg Ddu peninsula, a relation between high imposex levels and pollution sources can be speculated. Not only two mooring areas occur but also this peninsula is, at all extent, the District Golf Course (see below point 3.3.4.1). Therefore, pesticides might be used for molluscs or weed control possibly containing TBT or TPT compounds, which may be affecting the surrounding dogwhelk populations. However, the organotin analysis of the tissues (see point 3.3.3) revealed a very low level of TPT indicating that, at least for this compound, this was not the cause for the increasing values of imposex.

3.3.2 – Population's abundance

Numbers of animals per minute were calculated in order to give a very rough idea about the population's abundance at each sampling site. After collection, dogwhelks were counted and that number was divided by the minutes that the sampling lasted (Table 4). The mean number of animals collected per minute across stations was 11.7 (SD=7.2) in 2004/2005 and 16.3 (SD=8.7) in 2006. This suggests that dogwhelks are relatively abundant in the study area, which is expected since sterilization is almost negligible.

Table 4 – *Nucella lapillus*. Numbers of animals per minute in 2004/2005 and 2006 (n.a. – not assessed).

Sites		Abundance (animals per minute)	
		2004/2005	2006
Anglesey	Menai Bridge	30.4	28.0
	Beaumaris	4.6	12.6
	Penmon Point	6.2	8.4
	Red Warf Bay	5.2	6.3
	Moelfre	6.4	9.3
	Bull Bay	5.4	19.5
	Church Bay	9.2	18.3
	Hollyhead	6.6	7.2
	Trearddur Bay	11.7	6.6
	Rhosneiger	11.0	28.3
	Cable Bay	10.7	19.5
Lleyn Peninsula	Maen Dylan	9.0	2.7
	Penrhyn Nefyn	19.8	21.9
	Careg Ddu – site 1	8.8	30.5
	Careg Ddu – site 2	2.6	n.a.
	Careg Ddu – site 3	10.3	9.0
	Careg Ddu – site 4	10.4	16.3
	Careg Ddu – site 5	19.0	14.7
	Porthor – Whistling sands	5.5	8.45
	Aberdaron	8.0	n.a.
	Abersoch	10.8	22.2
	Shell Island	26.8	28.8

3.3.3 – Imposex versus organotin relationship

In the current study the female's TBT and TPT tissue contents, across stations, varied from 0.8 to 38.9 and from 0.5 to 2.1ng Sn/g dw, correspondingly. TBT represents the higher fraction of organotins in the tissues. Besides, MBT was not detected in any sample and DBT was only found at four sites with a maximum value of 6.2 and a minimum of 2.7ng Sn/g dw (Table 5). This fact supports the idea that recent TBT inputs continue to occur.

The levels of TBT tissue contamination observed in the current work are low comparing to the ones registered at other regions of UK and Europe. Values reported by Gibbs *et al.* (1987) for SW England in 1986 showed that TBT body burdens in *N. lapillus* varied between 15 and 259ng Sn/g dw. For the NE English coast, in 1998, Evans *et al.*

(1999) reported values ranging from 8 and 92ng Sn/g dw. Ruiz *et al.* (1998) found TBT dogwhelk body burdens between 51 and 974ng Sn/g dw for NW coast of Spain in 1996 whilst Galante-Oliveira *et al.* (2006) reported values between 23 and 138ng Sn/g dw in 2003 for the coast of Portugal.

Table 5 – Concentration of organotins (ng Sn/g dw) for the whole tissues across the sampling stations around North Wales. Monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), diphenyltin (DPT), triphenyltin (TPT), dioctyltin (DOT) and trioctyltin (TOT) average recovery rates (±St. Deviation) were 43.9±6.70, 89.6±4.08, 94.0±3.54, 80.5±8.11, 129.4±13.14, 122.38±15.02 and 157.2±51.80%, respectively.

Station Name	Station Code	MBT	DBT	TBT	DPT	TPT	DOT	TOT
Menai Bridge	1	<6.4	<2.3	6.59	<0.1	0.57	<5.5	<0.2
Beaumaris	2	<6.4	3.12	18.5	<0.1	0.80	<5.5	<0.2
Moelfre	5	<6.4	<2.3	3.39	<0.1	0.43	<5.5	<0.2
Bull Bay	6	<6.4	3.42	13.8	<0.1	0.64	<5.5	<0.2
Church Bay	7	<6.4	<2.3	2.76	<0.1	1.44	<5.5	<0.2
Hollyhead	8	<6.4	<2.3	1.27	<0.1	0.52	<5.5	<0.2
Treaddur Bay	9	<6.4	<2.3	1.33	<0.1	0.81	<5.5	<0.2
Cable Bay	11	<6.4	<2.3	2.08	<0.1	0.60	<5.5	<0.2
Nefyn	13	<6.4	<2.3	38.9	<0.1	0.86	<5.5	<0.2
Careg Ddu – L1	14	<6.4	6.18	26.9	<0.1	1.47	<5.5	<0.2
Careg Ddu – L2	15	<6.4	<2.3	10.8	<0.1	<0.06	<5.5	<0.2
Careg Ddu – L3	16	<6.4	<2.3	2.49	<0.1	1.32	<5.5	<0.2
Careg Ddu – L5	18	<6.4	<2.3	0.79	<0.1	0.76	<5.5	<0.2
Abersoch	21	<6.4	2.74	4.48	<0.1	2.06	<5.5	<0.2

VDSI versus TBT content and VDSI versus TPT content relationships observed in the current work are presented in Figure 16 and Figure 17, respectively. A significant correlation was obtained between TBT and VDSI ($r=0.87$, $p<0.001$), which upholds the widely accepted concept that TBT is the main cause of imposex in the wild populations. No relationship could be found between TPT and VDSI ($r=0.04$; $p=0.88$).

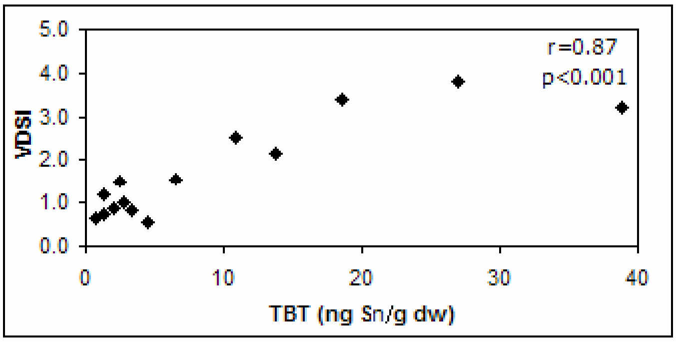


Figure 16 – *Nucella lapillus*. Relationship between TBT concentration in the tissues and VDSI.

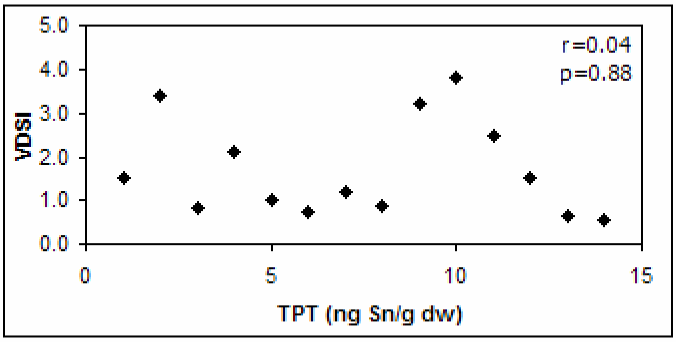


Figure 17 – *Nucella lapillus*. Relationship between TPT concentration in the tissues and VDSI.

3.3.4 – Temporal evolution of imposex in *Nucella lapillus*

Different authors assessed the imposex levels of *N. lapillus* around North Wales throughout the past two decades. Some of these authors surveyed the whole area - Meredith (1987), Oliveira (2005) - while others focused their studies mainly on the Isle of Anglesey - Dukes (1994), Hughes (1997), Saurel (2001) and Chatzinikolaou (2004) - or on the Lleyn Peninsula - Wagiman (1995). In order to simplify the temporal comparisons between the imposex data obtained in the current work and the data reported by the above authors, the study area is divided in two regions: the Anglesey and the Lleyn Peninsula (Table 6 and Table 7).

Table 6 – *Nucella lapillus*. Imposex data for 11 sites around Anglesey between 1987 and 2006: Female Penis Length Index (FPLI), Relative Penis Size Index (RPSI), Vas Deferens Sequence Index (VDSI) and percentage of affected females (%I). Standard Deviation (SD) is rounded off to decimal case and presented next to the mean value in the format mean (SD) (- not available; | not comparable).

Sites	Meredith 1987				Dukes 1994				Wagiman 1995				Hughes 1997			
	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I
Menai Bridge	-	34.500	5.2	100.0	1.70 (0.4)	8.916	3.4 (0.5)	100.0	2.33 (0.65)	5.740	3.6 (0.5)	100.0	1.91 (0.4)	6.030	3.7 (0.5)	100.0
Beaumaris	-	-	-	-	-	-	-	-	-	-	-	-	2.28 (0.9)	13.930	4.1 (0.3)	100.0
Penmon Point	-	6.300	3.5	100.0	2.67 (1.1)	0.782	2.7 (1.1)	91.6	1.40 (0.4)	1.030	3.2 (0.8)	100.0	-	-	-	-
Red Warf Bay	-	10.600	3.7	-	1.06 (0.4)	2.047	2.9 (0.3)	100.0	-	-	-	-	-	-	-	-
Moelfre	-	5.600	3.7	-	0.82 (0.5)	1.082	2.7 (0.8)	100.0	-	-	-	-	-	-	-	-
Bull Bay	-	11.000	4.1	-	0.92 (0.2)	1.613	3.2 (0.4)	100.0	-	-	-	-	-	-	-	-
Church Bay	-	4.800	3.5	-	2.62 (0.7)	0.672	2.6 (0.7)	100.0	-	-	-	-	-	-	-	-
Holyhead	-	16.800	4.2	-	0.85 (0.7)	1.330	2.00 (1.5)	66.7	-	-	-	-	-	-	-	-
Trearddur Bay	-	8.200	4.1	-	0.60 (0.5)	0.494	2.6 (1.0)	92.0	0.48 (0.6)	0.140	2.3 (0.8)	100.0	0.22 (0.3)	0.020	1.2 (1.2)	58.3
Rhosneiger	-	8.700	4.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Cable Bay	-	3.200	3.4	-	0.44 (0.4)	0.184	2.4 (0.9)	91.3	-	-	-	-	-	-	-	-

Sites	Saurel 2001				Chatzinikolaou 2004				Oliveira 2005				2006			
	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I
Menai Bridge	-	5.190	3.4	93.0	|	|	3.4	96.4	0.82 (0.7)	0.843	2.3 (0.8)|	|	0.25 (0.3)	0.020	1.6 (0.8)	97.4
Beaumaris	-	-	-	-	|	|	-	-	2.27 (0.8)	12.530	3.6 (1.0)|	|	1.60 (0.8)	2.530	3.4 (0.8)	100.0
Penmon Point	-	-	-	-	|	|	-	-	0.54 (0.5)	0.261	|	|	0.31 (0.5)	0.030	1.0 (1.2)	65.0
Red Warf Bay	-	-	-	-	|	|	-	-	0.40 (0.6)	0.097	|	|	0.45 (0.5)	0.075	1.7 (1.0)	93.0
Moelfre	-	-	-	-	|	|	-	-	0.17 (0.4)	0.010	|	|	0.08 (0.2)	0.000	0.8 (0.7)	64.5
Bull Bay	-	-	-	-	|	|	3.0	80.0	0.43 (0.5)	0.335	|	|	0.42 (1.9)	11.470	2.1 (1.0)	96.9
Church Bay	-	-	-	-	|	|	2.8	85.7	0.39 (0.4)	0.138	|	|	0.22 (0.4)	0.015	1.0 (1.0)	84.8
Holyhead	-	-	-	-	|	|	-	-	0.26 (0.3)	0.055	|	|	0.02 (0.1)	0.000	0.7 (0.5)	68.0
Trearddur Bay	-	-	-	-	|	|	2.4	29.2	0.18 (0.4)	0.010	|	|	0.20 (0.4)	0.009	1.2 (0.8)	83.3
Rhosneiger	-	-	-	-	|	|	-	-	0.03 (0.1)	0.003	|	|	0.12 (0.3)	0.003	0.9 (0.9)	61.1
Cable Bay	-	0.390	1.6	45.0	|	|	2.2	56.5	0.16 (0.4)	0.006	|	|	0.13 (0.2)	0.003	0.9 (0.9)	58.8

Table 7 – *Nucella lapillus*. Imposex data for 11 sites around Lleyn Peninsula between 1987 and 2006: Female Penis Length Index (FPLI), Relative Penis Size Index (RPSI), Vas Deferens Sequence Index (VDSI) and percentage of affected females (%I). Standard Deviation (SD) is rounded off to decimal case and presented next to the mean value in the format mean (SD) (- not available; | not comparable).

Sites	Meredith 1987				Dukes 1994				Wagiman 1995				Hughes 1997			
	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I
Maen Dylan	-	4.500	4.5	100.0	-	-	-	-	0.24 (0.5)	0.580	1.9 (0.9)	91.7	-	-	-	-
Penrhyn Nefyn	-	12.300	4.3	100.0	-	-	-	-	2.11 (0.9)	6.220	3.7 (0.5)	100.0	-	-	-	-
Careg Ddu – L 1	-	65.900	5.8	100.0	-	-	-	-	0.90 (0.5)	0.950	3.4 (0.5)	100.0	-	-	-	-
Careg Ddu - L 2	-	44.600	5.1	100.0	-	-	-	-	0.88 (0.4)	0.740	3.5 (0.5)	100.0	-	-	-	-
Careg Ddu - L 3	-	12.800	4.1	100.0	-	-	-	-	0.58 (0.6)	0.190	2.3 (1.4)	83.3	-	-	-	-
Careg Ddu - L 4	-	5.900	3.2	n.a.	-	-	-	-	0.11 (0.2)	0.002	1.6 (1.1)	80.0	-	-	-	-
Careg Ddu - L 5	-	3.000	2.8	n.a.	-	-	-	-	0.58 (0.8)	0.230	2.2 (1.1)	84.6	-	-	-	-
Porthor	-	1.200	2.4	n.a.	-	-	-	-	0.09 (0.2)	0.000	1.4 (1.1)	73.3	-	-	-	-
Aberdaron	-	0.700	1.8	n.a.	-	-	-	-	0.00 (0.0)	0.000	0.9 (0.9)	58.3	-	-	-	-
Abersoch	-	25.800	4.5	n.a.	-	-	-	-	1.69 (0.5)	3.730	3.8 (0.4)	100.0	-	-	-	-
Shell Island	-	n.a.	n.a.	n.a.	-	-	-	-	-	-	-	-	-	-	-	-

Sites	Saurel 2001				Chatzinikolaou 2004				2004/2005				2006			
	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I	FPLI	RPSI	VDSI	% I
Maen Dylan	-	-	-	-	-	-	-	-	0.00 (0.0)	0.000	|	|	0.00 (0.0)	0.000	1.0 (0.2)	92.6
Penrhyn Nefyn	-	-	-	-	-	-	-	-	1.35 (0.5)	4.926	|	|	1.20 (0.5)	1.435	3.2 (0.7)	100.0
Careg Ddu – L 1	-	-	-	-	-	-	-	-	1.42 (0.9)	7.900	|	|	1.78 (0.7)	4.624	3.8 (0.5)	100.0
Careg Ddu – L 2	-	-	-	-	-	-	-	-	0.46 (0.6)	0.141	|	|	0.80 (0.7)	0.468	2.5 (1.0)	100.0
Careg Ddu – L 3	-	-	-	-	-	-	-	-	0.34 (0.5)	0.054	|	|	0.22 (0.2)	0.013	1.5 (0.8)	93.3
Careg Ddu – L 4	-	-	-	-	-	-	-	-	0.05 (0.2)	0.068	|	|	-	-	-	-
Careg Ddu – L 5	-	-	-	-	-	-	-	-	0.03 (0.1)	0.000	|	|	0.05 (0.2)	0.000	0.6 (0.6)	56.2
Porthor	-	-	-	-	-	-	-	-	0.03 (0.1)	0.000	|	|	0.06 (0.2)	0.000	1.0 (0.7)	62.5
Aberdaron	-	-	-	-	-	-	-	-	0.15 (0.4)	0.006	|	|	-	-	-	-
Abersoch	-	-	-	-	-	-	-	-	0.00 (0.0)	0.000	|	|	0.00 (0.0)	0.000	0.5 (0.5)	48.9
Shell Island	-	-	-	-	-	-	-	-	0.12 (0.4)	0.002	|	|	0.02 (0.1)	0.000	0.6 (0.6)	54.3

The temporal variation of imposex at each site provides a reliable assessment of the evolution of TBT pollution in the area, which is very useful to evaluate the effectiveness of the legislation that restricts the use of organotin based AF paints in the UK. However, the comparison of imposex data between the different studies has to be done very carefully. In fact, it is important that the same methodology for assessing the imposex has been used and also that similar shell size (adult) animals have been sampled at each site. One of the major concerns regarding this last aspect is that penis length is known to be positively correlated with the size of the animal in both genders, which can severely affect the value of FPLI and RPSI. Besides, higher shell heights may denote older ages, which, considering the imposex irreversibility, may introduce some bias in the analysis. In this comparative analysis shell height data is only available for 1997 (Hughes, 1997) and 2004/2005 (Oliveira, 2005) surveys, for which no significant differences were found with our current study. In addition, the methodology used by each author should follow the same protocol. For instance, while most of the above authors used the methodology described by Gibbs *et al.* (1987), where the penis length is measured without narcotisation, Chatzinikolaou submitted the animals to magnesium chloride. Dogwhelks body tends to relax when narcotised and the penis becomes larger (Minchin and Davies, 1999), which influences the FPLI and RPSI values. Hence, we will use these indeces only to perform statistical comparisons between the current study and our previous survey as the above requirements were observed and also because VDSI for most of the stations was not assessed by Oliveira (2005).

The VDSI is an index that depend less on the size of the animals (providing that they are adults with about the same age) and on the narcotisation procedure and thus can be used with more confidence in all comparisons. Besides, VDSI is the most biological meaningful parameter as it shows how the female reproductive capability can be affected. For these reasons, the VDSI is here selected as the main index for long-term temporal comparisons with other author’s data.

3.3.4.1 – Long-term comparisons (from 1987 to 2006)

The Anglesey region

From the eleven sites sampled in 2006, nine are common to the surveys performed in 1987 by Meredith (1987) and in 1994 by Dukes (1994). As these studies provide the highest number of common sites with our study, they were chosen for the following comparisons. However, since Meredith's (1987) original data is not available, we could only test statistical significance differences between our data and Duke's (1994) data (α=0.05).

The reduction of VDSI is obvious and consistent for all sites that were compared between 1987, 1994 and 2006. The most affected site in 1987 was Menai Bridge (st. 1) with a VDSI of 5.2 and all the other stations presented VDSI values from 3.4 to 4.1. In 1994 the VDSI declined 21-52% across these stations as it ranged only between 2.0 and 3.5. This decline could result from the introduction of the first legislation measures in 1986 and 1987 (see point 3.1). In 2006 a continuous decrease is still observed because the VDSI values, for the same sites, ranged between 0.7 and 2.1, corresponding to a decrease of 34-70% since 1994; this decrease is statistically significant for all sampling sites (Table 8 and Figure 18). The decline observed in this latter period is higher than that observed between 1987 and 1994 and this can be due to the different time span between the surveys (respectively 12 and 7 years) or may reflect the introduction of the ban on the application of TBT AF paints on all boats in 2003 (see point 3.1), as it seems to had occurred at Menai Bridge (see below). Globally, percentages of VDSI decrease are about 52-83%, which indicates a clear recovery of dogwhelks populations in the last two decades. As a matter of fact, Meredith (1987) reported the occurrence of sterile females at almost all stations in 1987, whilst in 1994 no sterile females were reported by Dukes (1994) and, in 2006, only one was found at Beaumaris.

The evolution of RPSI (Table 6) also confirms the same tendency of imposex decline from 1987 until the present study. RPSI decreased consecutively at all sites in the three surveys from 1987 till 2006, with the single exception of Bull Bay, that registered an increase in RPSI from 1.6 to 11.5% between 1994 and 2006. However, as FPLI also decreased in this site during the same period, the rising of the RPSI was caused by a decrease in MPLI (male penis length index) probably due to the collection of smaller males in this occasion. Nevertheless, it is interesting to note that the %I at each site did not decrease so drastically as the other indices; this is expected because the intensity of imposex declined throughout the two decades to low levels of imposex but it still did not reach the value of zero for most of the animals, a condition that is expected only when TBT pollution will be negligible.

Table 8 – *Nucella lapillus***.** Number of observed females (N♀) and Vas Deferens Sequence Index (VDSI) with the respective Standard Deviation (SD)**.** Mann-Whitney U test values comparing VDSI between 1994 and 2006 are also provided. p stands for the significance of the test.

Sites	Station Code	Dukes 1994			2006			U-test	p
		N ♀	VDSI	SD	N ♀	VDSI	SD		
Menai Bridge	1	11	3.4	0.5	39	1.6	0.8	23.50	0.000
Penmon Point	3	12	2.7	1.1	22	1.0	1.2	68.00	0.001
Red Warf Bay	4	10	2.9	0.3	43	1.7	1.0	59.50	0.001
Moelfre	5	15	2.7	0.8	31	0.8	0.7	30.00	0.000
Bull Bay	6	18	3.2	0.4	29	2.1	1.0	90.00	0.000
Church Bay	7	8	2.6	0.7	33	1.0	1.0	59.50	0.003
Hollyhead	8	18	2.0	1.4	25	0.7	0.5	180.00	0.029
Trearddur Bay	9	25	2.6	1.0	36	1.2	0.8	123.50	0.000
Cable Bay	11	23	2.4	0.9	34	0.9	0.9	109.00	0.000

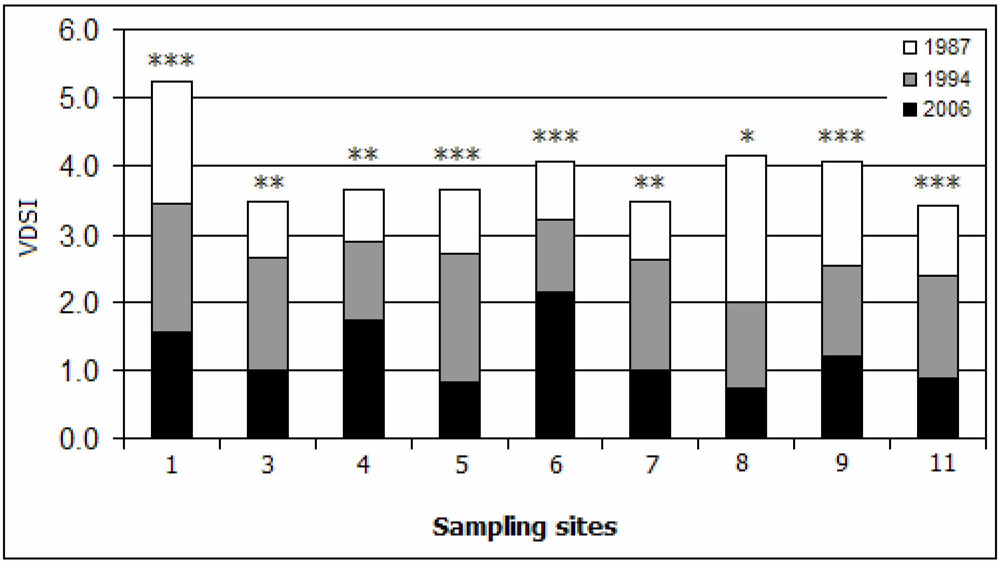


Figure 18 – *Nucella lapillus*. Vas Deferens Sequence index (VDSI) for each survey between 1987 and 2006. Significance of the statistical comparisons between 1994 and 2006 at common sites are represented by: * p<0.05; ** p<0.01 and *** p<0.001.

As several authors have studied the imposex of *N. lapillus* at Menai Bridge, it is possible to track the evolution of TBT pollution in this site with a more detailed temporal scale (Figure 19). In 1987 this place presented the highest VDSI of all surveyed area (5.2). This VDSI indicates the presence of sterile females in the population. Since then, imposex decreased considerably, possibly due to the first TBT ban (see point 3.1). From 1994 to 2004 the VDSI value remained approximately constant (near 3.5). Even though, no sterile females were observed and, during those years, this site continued to be the most affected site in Anglesey. In 2005 and 2006, VDSI decreased to respectively 2.3 and 1.6, probably as a response to the second TBT ban that entered into force in 2003 (see point 3.1). The drastic decrease of imposex at Menai Bridge from 1987 to 2006 is associated with a clear recovery of the population abundance; in the beginning of this period dogwhelks were rare (Berwyn personal communication) and, in 1994, Dukes (1994) reported timed searches estimates of 3.1 animals per minute whilst in 2006 we found the dogwhelks to be very abundant and we obtained timed searches estimates of 28 animals per minute. As referred above, the percentage of females affected by imposex did not show a so drastic decrease which means that TBT pollution is still affecting almost all the population: it varied from 100% (between 1987 and 1997) to 93.0% in 2001, but since then increased again and is recently 97.4%.

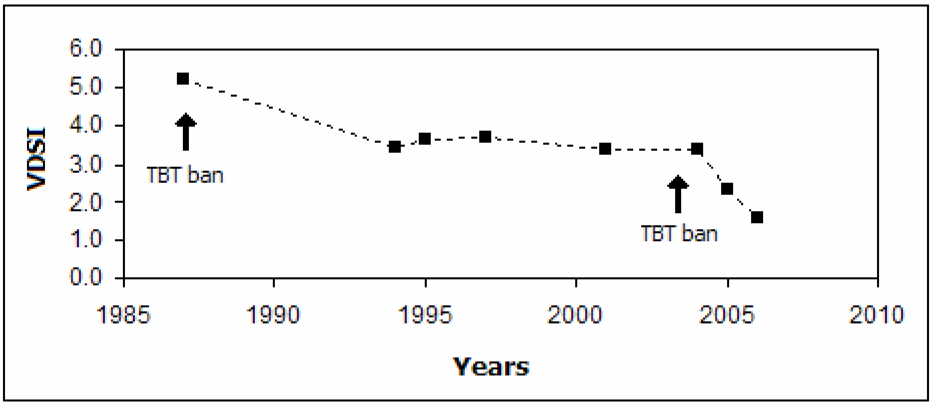


Figure 19 – *Nucella lapillus*. Vas deferens sequence index (VDSI) evolution between 1987 and 2006 at Menai Bridge with the indication of the time when bans on TBT AF paints were introduced in UK.

In 2006, Beaumaris was the most affected site in Anglesey (Table 6). This site was not included in the above comparisons because it was not sampled by Meredith (1987) or Dukes (1994) but it deserves to be analysed because this was the only place in Anglesey where we found female sterilization. The percentage of sterile females (%S) declined from 4.0% in 1997 (Hughes, 1997) to 1.8% in 2006, which is in accordance with the significant decrease of VDSI (U value=124.0 with $p<0.01$) during this period.

It is important to assess the global imposex evolution in Anglesey between 1987 and 2006 based on the nine sites common to Meredith (1987), Dukes (1994) and the current study. The high significance ($p<0.001$) of the Friedman test (Friedman – ANOVA =18.00) for VDSI indicates that there has been a clear recovery of dogwhelks' imposex in this region (Figure 20).

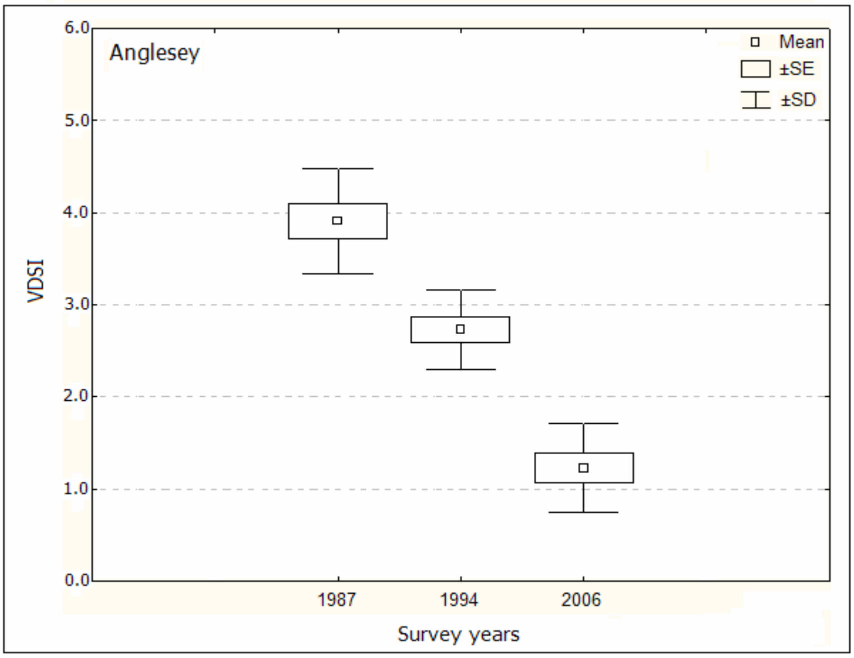


Figure 20 – *Nucella lapillus*. Vas Deferens Sequence Index (VDSI) mean, Standard Error (SE) and Standard Deviation (SD) for the surveys performed in 1987 (Meredith, 1987), 1994 (Dukes, 1994) and 2006 (current study) at the common sites around Anglesey.

Lleyn Peninsula

Among the nine sites sampled in 2006, eight are common to the surveys performed in 1987 by Meredith (1987) and in 1995 by Wagiman (1995). VDSI statistical comparisons are only possible with Wagiman's data (1995) because was the only author that provided detailed data.

A consecutive decrease in VDSI is generally observed for Lleyn Peninsula in the 1987 – 1995 and 1995 – 2006 periods. Nevertheless, the VDSI decrease seems not to be as consistent as in Anglesey since one site has shown an increase in VDSI values between 1995 and 2006 (Table 9 and Figure 21).

The most severely affected site in 1987 was Careg Ddu – L1 (st. 14) with a VDSI of 5.8, which was the highest value assessed in North Wales during the past 20 years and corresponds to a high incidence of sterile females. At the other sites the values ranged between 1.7 and 5.1. In 1995 VDSI declined 13-53% across stations and varied between 0.9 and 3.8. From 1995 until the present survey the decrease continued to occur and was statistically significant in 6 of the 8 sampling sites (Table 9 and Figure 21). In this period, the percentage of decline varied between 13 and 85%. The VDSI decline between 1995 and 2006 was higher than the one occurred between 1987 and 1995, probably due to the same reasons pointed above for Anglesey. Globally, percentages of VDSI decrease varied between 25 and 88% from 1987 until 2006.

The occurrence of sterile females in almost all stations of the Lleyn Peninsula in 1987 was reported by Meredith (1987) but no sterile females were reported by Wagiman (1995) and, in the current survey, only one occurred at Careg Ddu – L1.

Table 9 – *Nucella lapillus***.** Number of observed females (N♀) and Vas Deferens Sequence Index (VDSI) with the respective Standard deviation (SD)**.** Mann-Whitney U test results comparing VDSI between 1995 and 2006. p stands for the significance of the test.

Sites	Station Code	Wagiman 1995			2006			U test value	p
		N ♀	VDSI	SD	N ♀	VDSI	SD		
Maen Dylan	12	12	1.9	0.9	21	1.0	0.2	40.50	0.0008
Penrhyn Nefyn	13	15	3.7	0.5	47	3.2	0.7	213.00	0.0021
Careg Ddu – L 1	14	9	3.4	0.5	34	3.8	0.5	97.50	0.0981
Careg Ddu – L 2	15	14	3.5	0.5	37	2.5	1.0	170.50	0.0139
Careg Ddu – L 3	16	12	2.3	1.4	30	1.5	0.8	111.00	0.0557
Careg Ddu – L 5	18	13	2.2	1.1	32	0.6	0.6	63.00	0.0001
Porthor	19	15	1.4	1.1	32	1.0	0.7	151.50	0.0424
Abersoch	21	10	3.8	0.4	33	0.5	0.5	0.00	0.0000

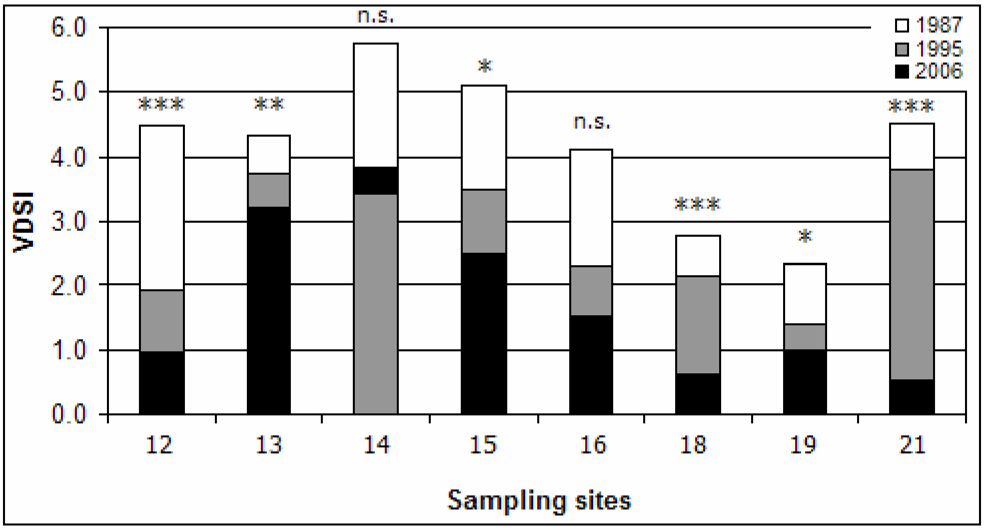


Figure 21 – *Nucella lapillus*. Vas Deferens Sequence Index (VDSI) for each survey between 1987 and 2006 at Lleyn Peninsula. Significance of the statistical comparisons between 1995 and 2006 at common sites are represented by: n.s. – not significant; * p<0.05; ** p<0.01 and *** p<0.001.

Careg Ddu – L1 was the single exception for the imposex decreasing trend described for the whole study area. In fact, it showed an increase of 11% in relation to 1995, which occurred not only in the VDSI (from 3.4 to 3.8) but also in the RPSI (from 1.0 to 4.6%) (Table 7) remaining, this way, the most affected site in North Wales in 2006. The increase of VDSI at this site was not statistically significant, anyway it deserves special attention because it could suggest a possible increase of naval traffic in the area (for which we have no data) or even an illegal use of TBT AF paints or other source of

TBT/TPT compounds. It should be noted that Meredith (1987) described a gradient of imposex nearby Careg Ddu peninsula that decreased from Cared Ddu L1 to L5 and from L1 to Penrhyn Nefyn. This same tendency around this peninsula can still be seen in 2006 even when imposex levels had decreased (Figure 22). The spatial imposex gradient in such a small area is related with the proximity of TBT pollution sources, two mooring places for small boats located at Porth Dinllaen and Nefyn (Figure 22 and Annex I). If there is an increase of naval traffic, or an illegal use of TBT AF paints in these places, then we would expect an increase in the imposex levels in the sites along the gradient. As this was not the case, we could only assume that the rising values of imposex at Careg Ddu – L1 are derived from random sampling error or to some rather localized additional input of TBT. As far as we know, there is no information reported by other authors regarding the organotin contamination of *N. lapillus* for previous years in North Wales and, for this reason, there is no way to assess any temporal decrease in the last two decades. Nevertheless, short-term comparisons between 2004/2005 and 2006 can give a further insight regarding this issue (see below point 3.3.4.2).

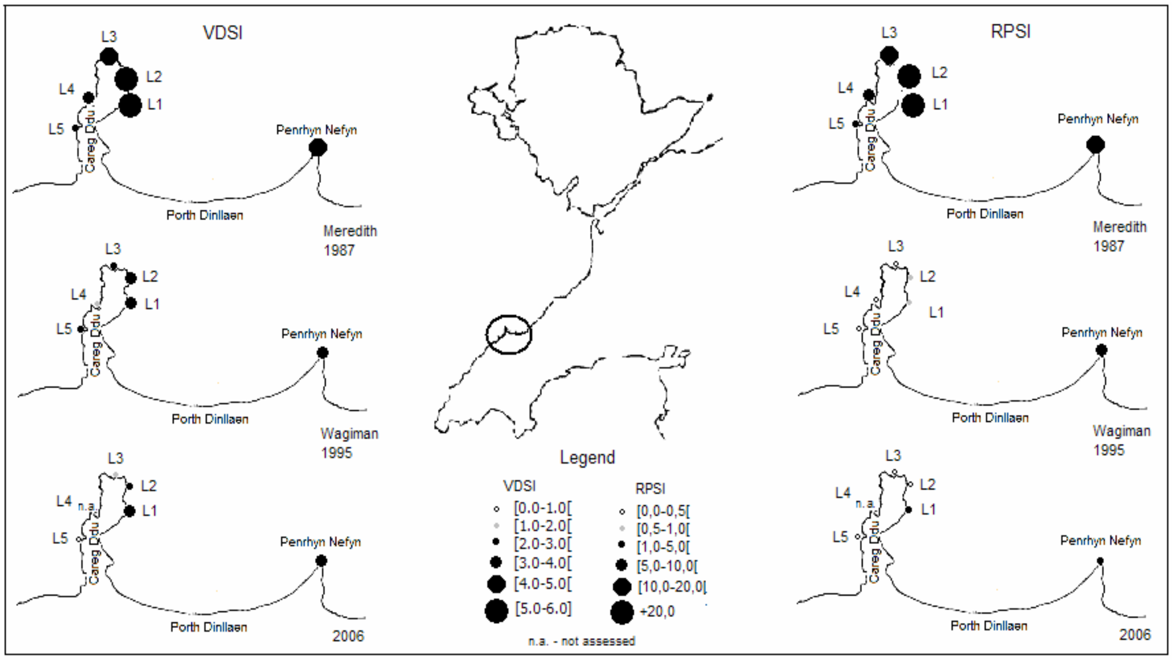


Figure 22 – Spatial gradient and temporal evolution of imposex indices: Vas Deferens Sequence Index (VDSI) and Relative Penis Size index (RPSI) around Careg Ddu and Penrhyn Nefyn between 1987 and 2006.

As it was observed for Anglesey, there has been also a global imposex decrease in the region of Lleyn Peninsula between 1987 and 2006 (Friedman – ANOVA test=14.25, $p<0.001$), based on the comparison of the VDSI data available for the eight sites common to Meredith (1987), Wagiman (1994) and the current study (Figure 23).

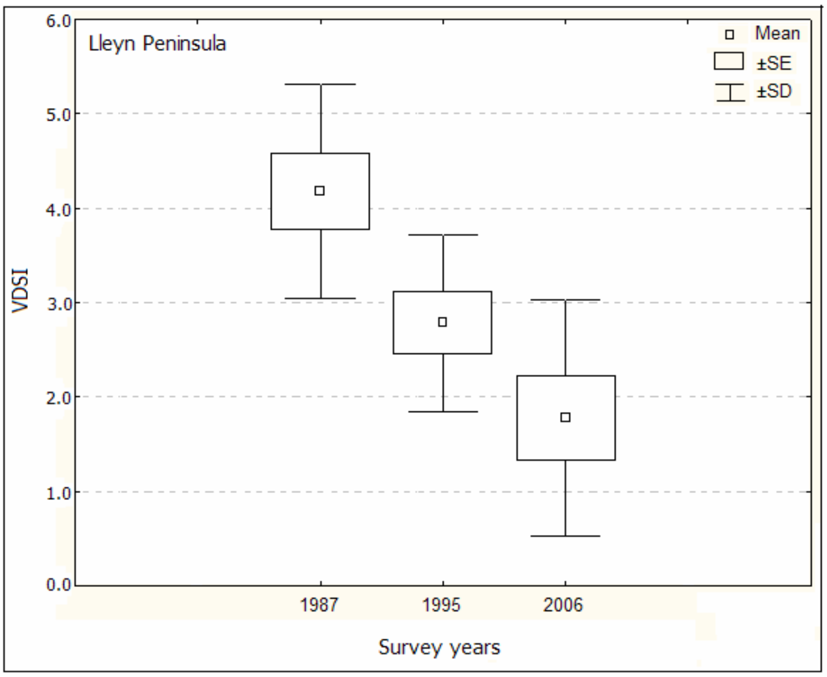


Figure 23 – *Nucella lapillus*. Vas Deferens Sequence Index (VDSI) with respective Standard Error (SE) and Standard Deviation (SD) for the surveys performed in 1987 (Meredith, 1987), 1994 (Dukes, 1994) and 2006 (current study) around Lleyn Peninsula.

3.3.4.2 – Short-term comparisons (2004/2005 and 2006)

Temporal comparisons between 2006 and 2004/2005 are based on the FPLI since there is no sufficient available data for VDSI in 2004/2005 (Figure 24). Shell heights demonstrated no statistical difference between both years, which validate the comparisons with the FPLI (Table 10).

Table 10 – *Nucella lapillus.* Number of assessed females (N♀), sean shell height (Shell Height), Female Penis Length Index (FPLI) and the Mann-Whitney U test significance (p) between FPLI (U value for FPLI) observed both in 2004/2005 and 2006: *** $p<0.001$; ** $p<0.01$; n.s.: non significant.

Site	2004/2005			2006			U value for FPLI	p
	N ♀	Shell Height (mm)	FPLI (mm)	N ♀	Shell Height (mm)	FPLI (mm)		
Menai Bridge	65	29.39	0.82	39	29.40	0.25	593.00	***
Beaumaris	56	30.88	2.27	36	31.50	1.60	514.00	***
Penmon Point	37	27.15	0.54	22	27.35	0.31	300.50	n.s.
Red Warf Bay	29	27.89	0.40	40	29.12	0.45	557.00	n.s.
Moelfre	27	28.54	0.17	31	29.31	0.08	389.00	n.s.
Bull Bay	30	27.60	0.43	29	28.83	0.42	421.00	n.s.
Church Bay	28	26.27	0.39	33	26.68	0.22	354.00	n.s.
Hollyhead	27	28.88	0.26	25	29.27	0.02	195.00	**
Trearddur Bay	34	28.24	0.18	36	28.58	0.20	576.50	n.s.
Rhosneiger	34	26.28	0.03	36	26.31	0.12	514.00	n.s.
Cable Bay	44	27.45	0.16	34	26.58	0.13	713.00	n.s.
Maen Dylan	41	24.90	0.00	21	23.84	0.00	430.50	n.s.
Penrhyn Nefyn	33	28.42	1.35	47	28.43	1.20	661.50	n.s.
Careg Ddu - L1	49	29.48	1.42	34	28.87	1.78	713.50	n.s.
Careg Ddu - L2	45	29.56	0.46	37	28.99	0.80	544.50	***
Careg Ddu – L3	40	28.37	0.34	30	28.24	0.22	590.50	n.s.
Careg Ddu - L5	37	29.50	0.03	32	28.86	0.05	585.00	n.s.
Porthor	32	28.62	0.03	32	28.44	0.06	495.00	n.s.
Abersoch	39	28.30	0.00	34	28.54	0.00	663.00	n.s.
Shell Island	50	29.21	0.12	46	28.75	0.02	1058.0	n.s.

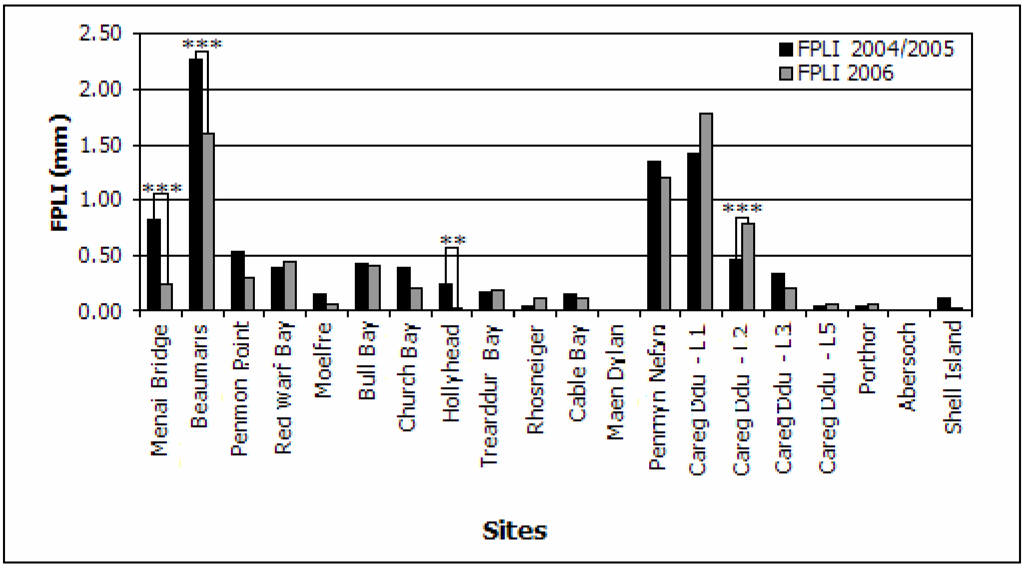


Figure 24 – *Nucella lapillus*. Evolution of Female Penis Length Index (FPLI) between 2004/2005 and 2006 around North Wales. Significance was achieved using a Mann-Whitney U test and represented by: *** p<0.001; ** p<0.01. Pares of values without * have no statistical significance.

In general, a slight decline in FPLI values can be noticed between 2004/2005 and 2006, but no significant difference was observed between the two studies for the whole North Wales surveyed area (W value=0.71, p=0.48), probably due to the very short time span that is being compared. Menai Bridge, Beaumaris and Hollyhead showed a significant decrease of imposex but, on the other side, Careg Ddu L2 showed a significant increase. In addition, Careg Ddu – L1 showed a slight increase of FPLI which suggests that possibly a localized increase of TBT pollution may had really happened next to these sites, which should be further investigated in the future (see point 3.3.4.1).

3.4 – Conclusions

Imposex levels in North Wales have been significantly reduced since 1987 following the introduction of legislation restricting the commercialization and usage of TBT based AF paints in the UK. The reduction of the organotin content in AF paints in 1986 and the first ban targeting pleasure crafts and marine aquaculture equipment in 1987 leaded to a first recovery of dogwhelks' populations throughout North Wales. This recovery was initially rapid but, as revealed by what happened at Menai Bridge, probably it levelled out in the following years (1994 and 1995 surveys) showing that further action, such as a complete ban on TBT, was probably necessary to reduce TBT pollution. After the EU 2003 ban, ships could not re-paint their hulls with TBT based AF paints. This fact may have contributed to the further decrease on imposex levels observed in the most recent years. The imposex decrease lately observed between 2004/2005 and 2006 do not include all surveyed area; at a localized spot at Careg Ddu L1 and L2 there was an increase in imposex (see point 3.3.4.2) showing that probably there was either a rising in the naval traffic, illegal uses of TBT AF paints or any other different source of TBT.

To our best knowledge, there is no information reported by other authors regarding the organotin contamination of *N. lapillus* for previous years in North Wales and, for this reason, there is no way to assess any temporal decrease in the last two decades. However, the tissue organotin levels detected in 2006 for the study area are very low comparing to the values reported in the past for other regions of UK and other European countries (see point 3.3.3). This fact is in accordance with the great reduction of the imposex levels observed for *N. lapillus* in North Wales. Despite this reduction, there are evidences that TBT based AF paints are still being used – at least in old painted ship hulls – and that fresh TBT inputs continue to occur.

3.5 – References

Abel R, Hathaway RA, King NJ, Vosser JL, Wilkinson TG (1987). Assessment and regulatory actions for TBT in the UK, Oceans '87. Conference Record 4: 1314 - 1319.

Chatzinikolaou E (2004). The ecology and growth of the netted dogwhelk *Nassarius reticulatus* (Gastropoda: Nassariidae). PhD thesis, School of Ocean Sciences, University of Wales, Bangor.

Cleary JJ (1991). Organotin in the marine surface microlayer and sub-surface waters of south-west England: relation to toxicity thresholds and the UK Environmental Quality Standard. Marine Environmental Research 32: 213 - 222.

Dukes MJ (1994). Assessment of the recovery from the effects of tributyltin pollution observed in *Nucella lapillus (L.)* populations around the coast of Anglesey, North Wales. BSc honours project, School of Ocean Sciences, University of Wales, Bangor.

Evans SM, Kerrigan E, Palmer N (2000). Causes of Imposex in the dogwhelk *Nucella lapillus* (L.) and its use as a Biological Indicator of Tributyltin Contamination. Marine Pollution Bulletin 40 (3): 212 - 219.

Galante–Oliveira S, Langston WJ, Burt GR, Pereira ME, Barroso CM (2006). Imposex and organotin body burden in the dog-whelk (*Nucella lapillus* L.) along the Portuguese coast. Applied Organometallic Chemistry 20: 1 - 4.

Gibbs PE, Bryan GW (1987). The Paints and the demise of the dog whelk, *Nucella lapillus* (Gastropoda). Journal of the Marine Biological Association of the United Kingdom 19: 1482 - 1487.

Google maps - [cited 11/11/2007]. Available from: http://maps.google.com/

Hughes C (1997). Variations in the Levels of Tributyltin (TBT) induced imposex in the dogwhelk, *Nucella lapillus*, along the Menai Strait. MSc dissertation, School of Ocean Sciences, University of Wales, Bangor.

Meredith LE (1987). Tributyltin induced imposex in populations of the dogwhelk *Nucella lapillus* in North Wales. MSc thesis, School of Ocean Sciences, University of Wales, Bangor.

Minchin A, Davies I (1999). Imposex measurement in the dogwhelk *Nucella lapillus* (L.) - temporal aspects of specimen preparation. Journal of Environmental Monitorization 1: 239 - 241.

Oliveira I (2005). Recovery from imposex in populations of the common dogwhelk, *Nucella lapillus*, around Anglesey and Lleyn Peninsula, North Wales. Project Study, Universidade de Aveiro, Portugal.

OSPAR (1998). Joint Assessment and Monitoring Programme (JAMP), Guidelines for contaminant specific biological effects monitoring, Ref. Nº 1998 – 3.

Ruiz J, Quintela M, Barreiro R (1998). Ubiquitous imposex and organotin bioaccumulation in gastropods *Nucella lapillus* (L.) from Galicia (NW Spain): A possible effect of nearshore shipping. Marine Ecology Progress Series 164: 237 - 244.

Saurel C (2002). An investigation into the use of statoliths for determining the age and growth rate of dogwhelks *Nucella lapillus* (L.) in North Wales. MSc dissertation, School of Ocean Sciences, University of Wales, Bangor.

Sokal RR, Rohlf FJ (1987). Introduction to Biostatistics. 2nd edition Freeman VH & Company, New York.

Sousa A, Matsudaira C, Takahashi S, Tanabe S, Barroso C (2007). Integrative assessment of organotin contamination in a southern European estuarine system (Ria de Aveiro, NW Portugal): Tracking temporal trends in order to evaluate the effectiveness of the EU ban. Marine Pollution Bulletin 54: 1645 - 1653.

Wagiman SB (1995). Recovery in the levels of imposex in Population of Dogwhelks. MSc Thesis, School of Ocean Sciences, University of Wales, Bangor.

Waldock MJ, Thain JE, Waite ME (1987). The distribution and potential toxic effects of TBT in UK estuaries during 1986. Applied Organometallic Chemistry 1: 287 - 301.

Chapter 4 – Final remarks

The current work has confirmed that *Nucella lapillus* imposex is caused by TBT pollution. In fact, when females were exposed in the laboratory to TBT, they developed higher levels of imposex and besides, significant correlations were observed in the field between the TBT content in tissues and the imposex degree. These evidences uphold, once more, that the intensity of imposex development depends on the degree of TBT contamination. Moreover, there seems to be a reasonable geographical uniformity in this dose-response relationship, which makes *N. lapillus* a good species to be used as an indicator of TBT pollution. The utility of this species to monitor this kind of pollution was proved in the field survey carried out in 2006 around North Wales. Actually, this survey helpeded to understand the intensity and spatial distribution of TBT pollution in North Wales. This pollution is spread all over the coast with relatively low levels. On the other hand, it clearly showed that there was a reduction of TBT pollution in the area in the last two decades, probably as a consequence of the legislative restrictions applied in UK regarding the use of organotin based AF paints. Despite this reduction, fresh TBT inputs continue to occur since the OT contamination in the dogwhelks tissues is largely dominated by TBT. After September 2008 the circulation of ships painted with organotin based AF paints will be forbidden and, hopefully, TBT pollution will cease.

Annex I

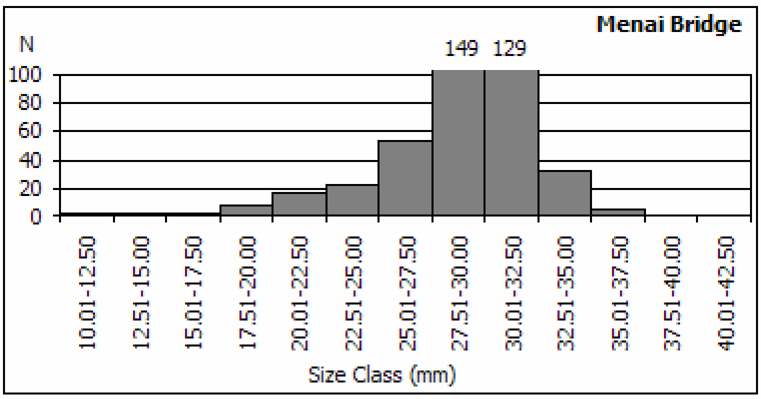
In this Annex is provided a general description of each sampled site regarding its location, physiography, dominant organisms' coverage, hydrodynamism and local human activities, with particular relevance to boat traffic – ports, marinas, mooring areas, etc. It is also presented, for each site, a histogram with the number and size of *Nucella lapillus* caught for the timed searches periods of 15 minutes. These histograms provide a rough indication of the dogwhelks abundance.

Menai Bridge

Figure 25 – Photograph of the sampling site (left) and its location (right). Mature and juvenile dogwhelks could be easily collected on the surface of the rocks.

Menai Bridge is a small town situated on the shores of the fifteen mile long Menai Strait on the Isle of Anglesey, North Wales. Much of the Strait is bordered by steep wooded banks with sand and mud flats at either end. The sampling site (Figure 25) is situated on a sheltered rocky area in a place where the Strait is relatively narrow and the currents are very strong (5-6 knots on spring tides). Dogwhelks were surveyed under the Suspension Bridge crossing the Menai Strait and were found together with egg capsules in great abundance all over the shore (mainly in crevices and under boulders) often with mature adults on the open rock. The algae were very abundant and were mainly composed of fucoid algae and kelps.

Since tourism is a major contributor to the local economy, water leisure activities such as sailing, jet skiing and scuba–diving are very popular in the Menai Strait (Saurel, 2002). There is also a wood yard situated about 800 metres north that is believed to be a possible source of TBT pollution into the Menai Strait (Wagiman, 1995).



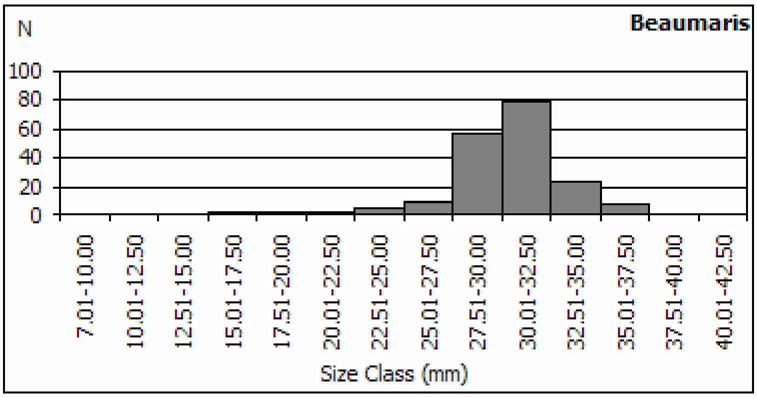
Total number of animals collected during the 3 timed searches: 420.

Histogram 1 – Menai Bridge. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Beaumaris

Figure 26 – Photograph of the sampling site (left) and its location (right). Sampling was undertaken on the small boulders seen in the picture close to the water edge.

Beaumaris is a small town on the Isle of Anglesey, situated on the shores of the Menai Strait and relatively near to Menai Bridge. The sampling site was located on a sheltered area with very small boulders covered with fucoid algae (Figure 26), at a place where the Strait is wide and the currents are very strong. It is situated next to the town's Pier. Boating activity in this area is very intense and at Gallows Point there is a large marina, which may constitute major sources of TBT pollution in this area.



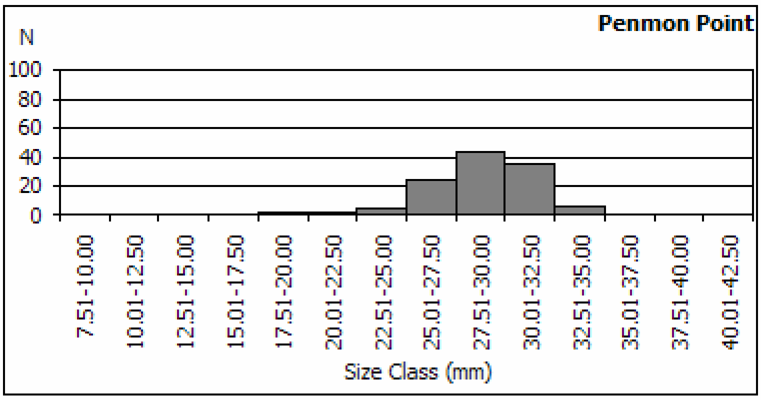
Total number of animals collected during the 3 timed searches: 189.

Histogram 2 – Beaumaris. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Penmon Point

Figure 27 – Photograph of the sampling site (left) and its location (right). Dogwhelks were collected mainly in the boulders' crevices which can be seen in the foreground of the picture.

Penmon Point is situated at the Northeast end of the Menai Strait, in front of Puffin Island, an uninhabited private island off the east coast, which is a special protection area to cormorant colony along with other birds. It is a popular tourist attraction on Anglesey and boat trips are frequently made around this place. The sampling site is located next to the light house, on the boulder shore (Figure 27). This narrow headland is moderately exposed to wave action, so dogwhelks were mainly found in crevices. Fucoid algae, mussels and barnacles were abundant.



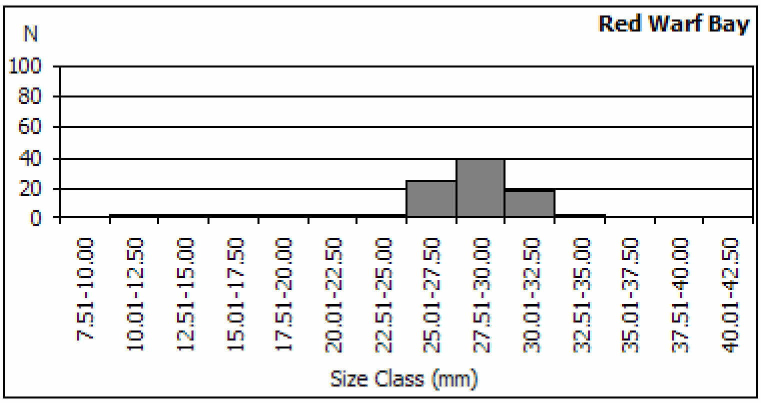
Total number of animals collected during the 3 timed searches: 126.

Histogram 3 – Penmon Point. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Red Warf Bay

Figure 28 – Photograph of the sampling site (left) and its location (right). Dogwhelks were collected on the rocks shown on the left part of the picture.

Red Wharf Bay is a large bay situated on the Northeast coast of Anglesey. This bay is very shallow at low tide where almost 25 km^2 of sand stays uncovered. This usually sheltered bay is bordered by salt marshes and sand dunes and attracts tourists during the Summer. In some occasions the shore becomes wave-exposed during north easterly winds and storms. Dogwhelks were found in the crevices of the rocks that bordered the beach (Figure 28). Only a few immature whelks and egg capsules were found on this site. Boating activity is negligible.



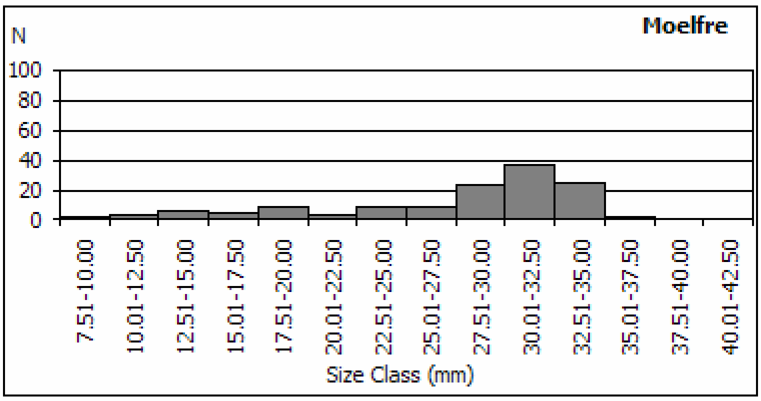
Total number of animals collected during the 3 timed searches: 94.

Histogram 4 – Red Warf Bay. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Moelfre

Figure 29 – Photograph of the sampling site (left) and its location (right) Dogwhelks were collected in this area and were easily found beneath boulders or on the surface of the rocks.

Molfre is a village on the north-east of Anglesey. The shore is a sheltered rocky beach with small boulders and only rarely exposed to easterly winds which makes this area a good place to moor boats. In fact, some boats were moored in the bay next to the shore where the survey was carried out. However is in the Summer that a major presence of boats can be regular. This may suggest that the dogwhelk population could have been or is still affected by TBT pollution. The rocky shore was extensively covered with algae as can be seen in Figure 29. Barnacles, *Semibalanus balanoides*, were abundant while mussels were found in a lower extent.



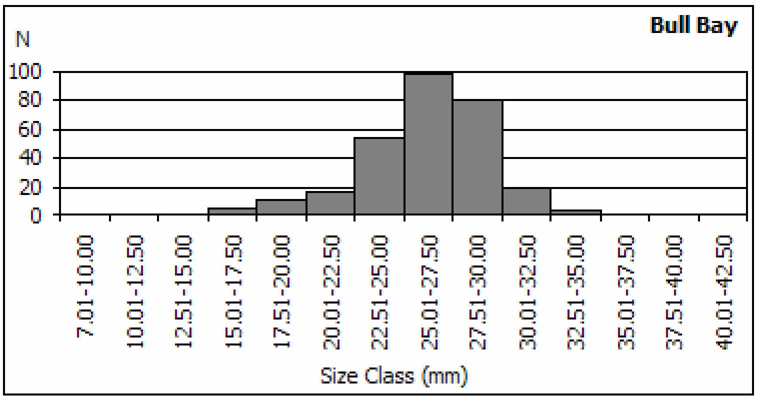
Total number of animals collected during the 3 timed searches: 140.

Histogram 5 – Moelfre. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Bull Bay

Figure 30 – Photograph of the sampling site (left) and its location (right). Dogwhelks were collected on both sides of the shore.

Bull Bay is a small village on the north coast of Anglesey, Wales. The sampling site was located on a rocky shore adjacent to the village. This place is an exposed shore where dogwhelks could be found mainly in rock crevices. Only a few juveniles were found and hidden in crevices and in inaccessible places on the rocks. The rock surface was mainly covered by barnacles, fucoid algae and kelps (Figure 30). In the Summer many small boats can be seen anchored in the Bay. Holyhead, the major harbour on the island, is also relatively near. Many boats that sail to Manchester make their route by the north of Anglesey and so, it is expected that this site may be subjected to some pollution.



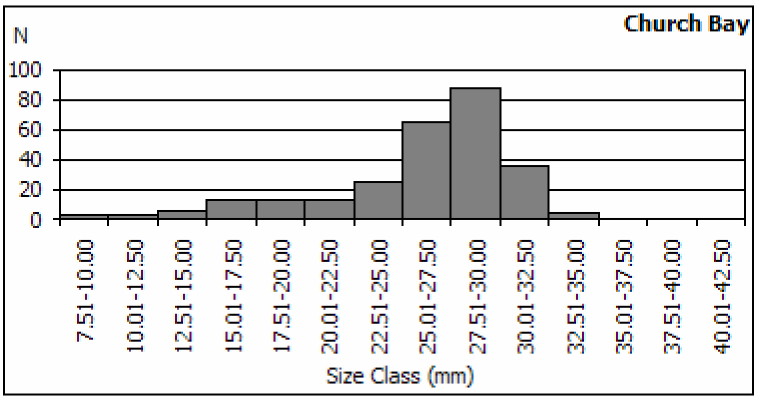
Total number of animals collected during the 3 timed searches: 293.

Histogram 6 – Bull Bay. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Church Bay

Figure 31 – Photograph of the sampling site (left) and its location (right). Dogwhelks were collected from the sheltered rocky pools and on the rocks seen in the picture.

Church Bay is an enclosed cove with sandy, pebbly beach with rock pools on the western north of the isle of Anglesey (Figure 31). This sampling site is exposed to southerly winds and dogwhelks were easily found on the surface of the rocks or in rock pools. Barnacles and mussels were present as well as some fucoid algae and kelps. Boating activity is negligible in this beach. However it is not too far from Hollyhead harbour.



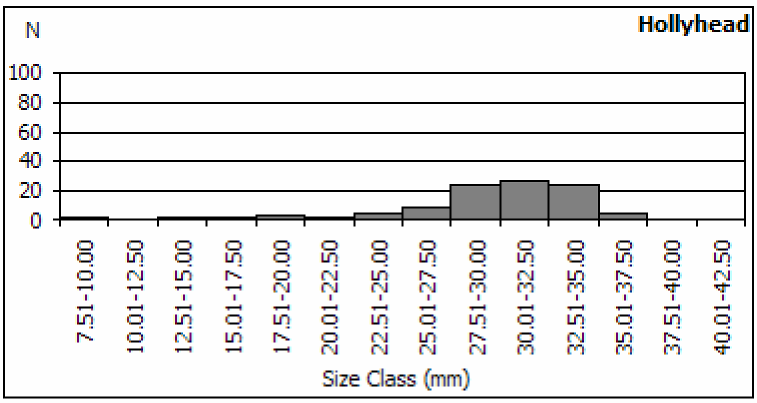
Total number of animals collected during the 3 timed searches: 275.

Histogram 7 – Church Bay. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Hollyhead

Figure 32 – Photograph of the sampling site (left) and its location (right). The majority of the dogwhelks were collected at the rocks next to the water but some animals were found under the boulders seen in the picture.

Holyhead is the largest town on the island of Anglesey and is well known by its busy ferry port. The sampled site is located on the north part of Hollyhead on the other side of the sea wall opposite to Soldiers Point (Figure 32). Only a few juveniles and egg capsules were found. Barnacles could be seen in great numbers. Dogwhelks were found under the boulders but were more abundant on crevices and even at the surface of rocks mainly covered with fucoid algae. This place is extremely exposed to wave action. High TBT levels are expected since the boating activity in this area is exceptionally high.



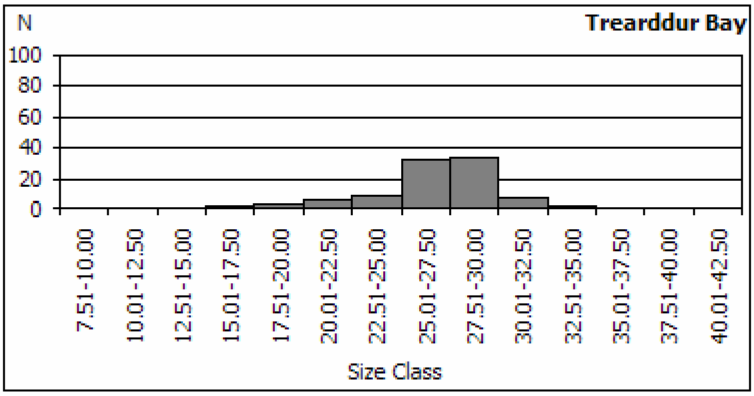
Total number of animals collected during the 3 timed searches: 108.

Histogram 8 – Hollyhead. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Trearddur Bay

Figure 33 – Photograph of the sampling site (left) and its location (right). Dogwhelks were collected in this area mostly on the surface of the rocks. Some animals were also covered by algae.

Trearddur Bay is located on the West coast of Anglesey, on the Holly Island, near Hollyhead. It is a well known resort for its exceptional beaches. The survey was carried out on the rocky shores and dogwhelks were easily found on the open rocks surface and rock pools (Figure 33). Egg capsules were abundant and found near to adult *Nucella lapillus* specimens, in crevices or under the algae. TBT pollution is not believed to be very high on this moderately exposed shore but, since Holyhead is the major harbour on the island, it is also expected that this site might experience some pollution (Saurel, 2002). The beach is popular for the tourists and small boating activity is very common in the area.



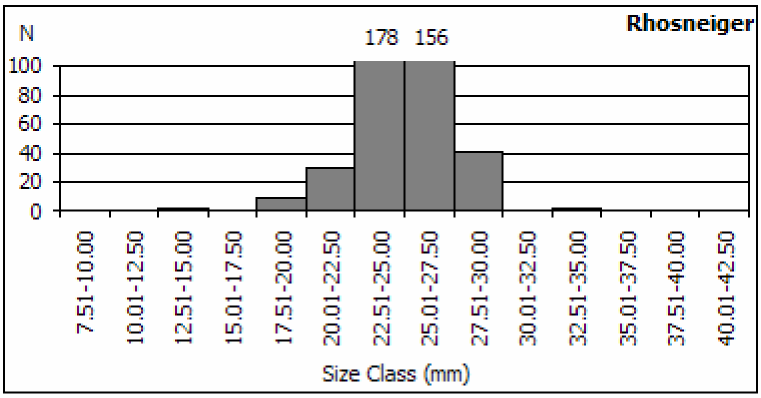
Total number of animals collected during the 3 timed searches: 99.

Histogram 9 – Trearddur Bay. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Rhosneiger

Figure 34 – Photograph of the sampling site (left) and its location (right). Sampling was carried out on the rocky pools and on the open rocks.

Rhosneiger village is situated on the west coast of Anglesey, North Wales - south from Trearddur Bay beach. The study site is located on a sandy beach with rock pools, in a great bay (Figure 34). This is a sheltered place with dogwhelks found abundantly on the rocks sometimes covered by fucoid algae. Plenty of egg capsule were also found. The rock surfaces were mainly covered by barnacles. Mussels could also be found on the lower part of the rocks. In this area, boating activity is negligible.



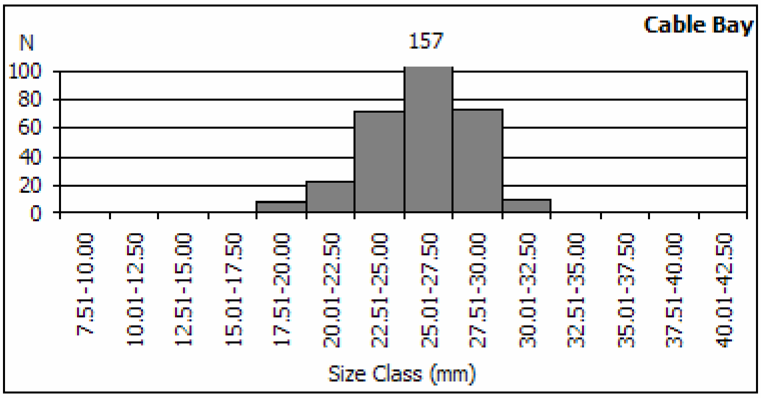
Total number of animals collected during the 3 timed searches: 424.

Histogram 10 – Rhosneiger. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Cable Bay

Figure 35 – Photograph of the sampling site (left) and its location (right). Collection was carried out on this rocky shore where the majority of the animals were easily found on the open rock.

Cable bay is situated on the southwest coast of Anglesey. This beautiful bay in the Summer can be a wild place in the Winter. It is exposed to prevailing west and south west winds, although there is less wave length than in other parts of the coast. The survey was carried out on the south coast, along the rocky shore in the bay (Figure 35). Dogwhelks were found on boulders and rocky surfaces, as well as under the algae cover, mainly constituted by fucoid algae and kelps. Barnacles were also present. Juveniles and egg capsules were rare and only found in hidden crevices. Boating activity is considered to be rare (Saurel, 2002).



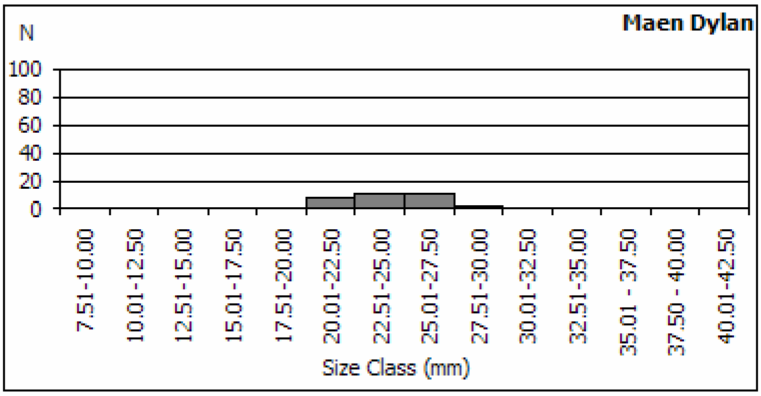
Total number of animals collected during the 3 timed searches: 293.

Histogram 11 – Cable Bay. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Maen Dylan

Figure 36 – Photograph of the sampling site (left) and its location (right). The few dogwhelks found were hidden in the rock crevices.

Maen Dylan is located in the south of Caernarfon on the Lleyn Peninsula, mainland North Wales. Boating activity close to Maen Dylan can be considered as sporadic during the Summer months (Saurel, 2002). This site is south west facing and moderately exposed to wave action (Figure 36). Dogwhelks were scarcely found as they were hidden in crevices or beneath boulders. Barnacles were abundant. No egg capsules or juveniles were found and the sampling was very hard to accomplish.



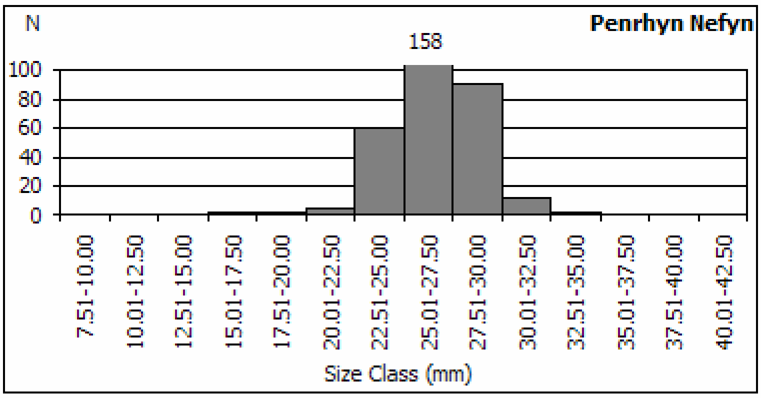
Total number of animals collected during the 3 timed searches: 40.

Histogram 12 – Maen Dylan. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Penrhyn Nefyn

Figure 37 – Photograph of the sampling site (left) and its location (right). Rocky promontory next to the small port where dogwhelks were found.

Penrhyn Nefyn is situated on the Lleyn Peninsula, north from the small Careg Ddu peninsula. The sampling site is situated on a rocky promontory between two beaches where boat activity is known to be very common. Several boats were seen anchored in this minor port. The shore is constituted by Precambrian igneous rocks, dominated by fucoid algae and barnacles (Wagiman, 1994). Mussels were rare and only present in the lower part of the shore. Dogwhelks were easily found on the open rocks surface, under the algae and in crevices (Figure 37). Both adults and juveniles were effortlessly found in this rocky shore. Egg capsules and juveniles were also abundant.



Total number of animals collected during the 3 timed searches: 329.

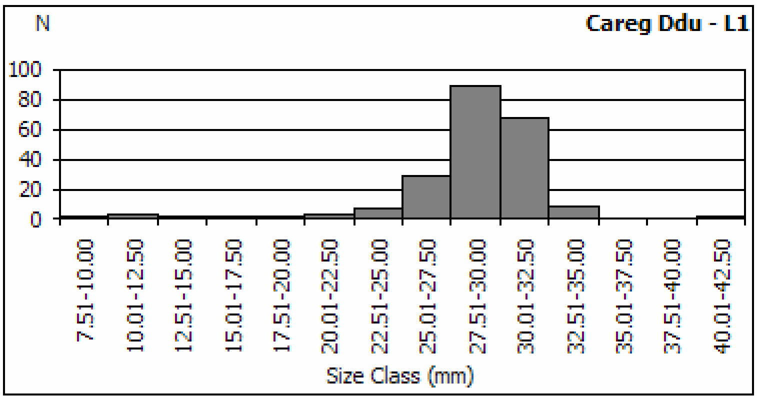
Histogram 13 – Penrhyn Nefyn. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Careg Ddu

Figure 38 – Location of the Careg Ddu Peninsula (upper picture) and photographs of the five sampled sites where dogwhelks were collected.

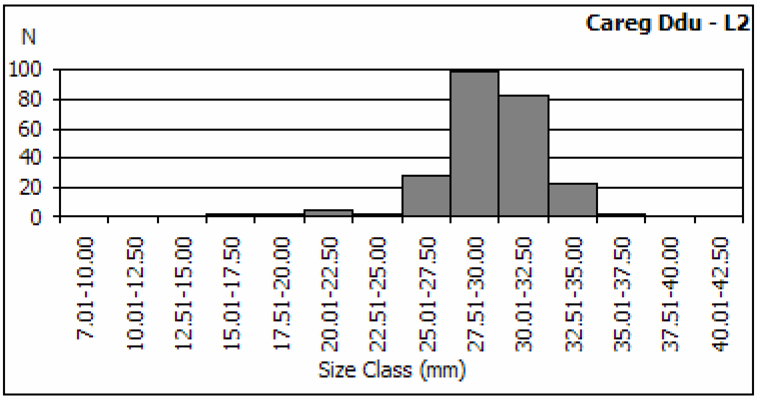
Careg Ddu is a small peninsula situated in Caernarfon Bay on the north western coast of the Lleyn Peninsula, adjacent to a private golf course. This small peninsula, of approximately 1km long and 200m wide, has a wide range of habitats, including rocky and sandy beaches ranging from sheltered to expose. Population surveys were conducted at 5 sites chosen for their different characteristics (Figure 38).

The north eastern side, where Locations 1 and 2 are both situated, formed sheltered bays. At both sites, dogwhelks were found on rocks dominated by the presence of barnacles but no mussel populations were observed during the sampling. Careg Ddu – L1, was the closest to the small village anchorage area. Several boats were moored in the bay and, in the Summer, tend to increase in number. In 1995, Wagiman described a sewage outfall which was discharging screened macerated sewage located 300m from the fifth sampling site (Wagiman, 1995). Careg Ddu - L2 is situated between the tip of the peninsula (L3) and L1. This is a sheltered site and the survey took place on the rocks seen in the respective picture of Figure 38. Dogwhelks were abundant and easily collected on open surfaces or beneath algae (usually kelps). Egg capsules were found at both sites.



Total number of animals collected during the 3 timed searches: 221.

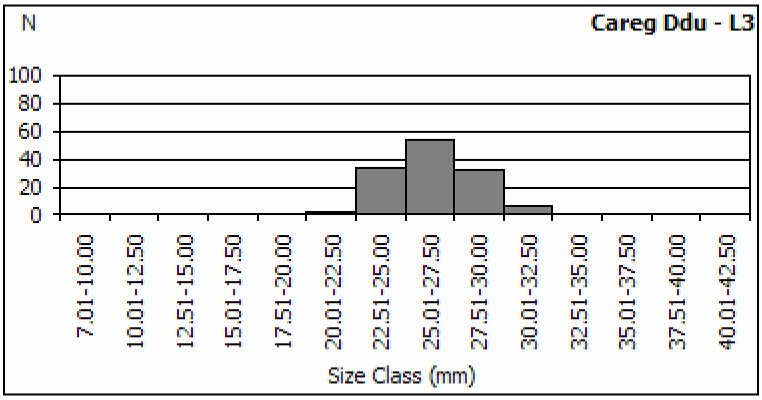
Histogram 14 – Careg Ddu – L1. Total number of dogwhelks caught in the three timed searches and number of animals for size class.



Total number of animals collected during the 3 timed searches: 245.

Histogram 15 – Careg Ddu – L2. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

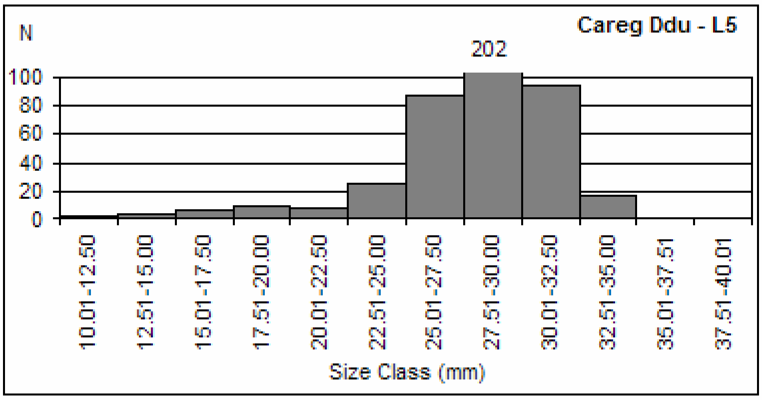
Careg Ddu – L3 (Figure 38) is situated right on the tip of the peninsula where the rocky shore is extremely exposed to the wave action. Specimens were found on vertical rocky surfaces, in crevices and under the boulders. Egg capsules were not abundant and generally hidden in rocks crevices. Barnacles were very abundant as well as fucoid algae.



Total number of animals collected during the 3 timed searches: 135.

Histogram 16 - Careg Ddu – L3. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

The western side of the peninsula shore is comprised of Precambrian rocks where sampling sites Careg Ddu – L4 and L5 are situated (Wagiman, 1995). Both sites are normally exposed to wave action but, even though, they have some differences. In 2004/2005, at the fifth location, both mature and juvenile dogwhelks were easily collected in crevices and on the rocks surface, while in the fourth location dogwhelks were uncommon and only adults were found. In the present survey, we decided to sample only in Careg Ddu – L5 since it was easier, the animals were more abundant and it was not so dangerous. At both sites barnacles were small and sparse but mussels were easily found.



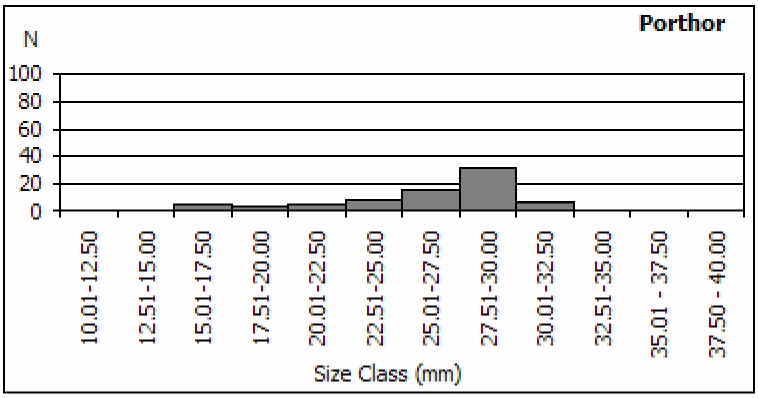
Total number of animals collected during the 3 timed searches: 458.

Histogram 17 – Careg Ddu – L5. Total number of dogwhelks caught in the three timed searches and number animals for size class.

Porthor

Figure 39 – Photograph of the sampling site (left) and its location (right). Sampling took place on the rocky promontory that protected the beach.

Porthor is a beautiful, remote sandy beach at the north western end of the Lleyn Peninsula (Figure 39). The English name Whistling sands is derived from the whistle emitted by the peculiar shaped sand particles being rubbed together when walked on. This popular beach adjoins a fine stretch of National Trust owned coastline which attracts many tourists during the summer. There is no pollution input known in this area. This beach is fairly sheltered and protected from wave action by rocky promontories. Dogwhelks were found on the rockcs surface and in crevices. Barnacles dominated the entire shore as well as kelps and fucoid algae.



Total number of animals collected during the 3 timed searches: 82.

Histogram 18 – Porthor. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Aberdaron

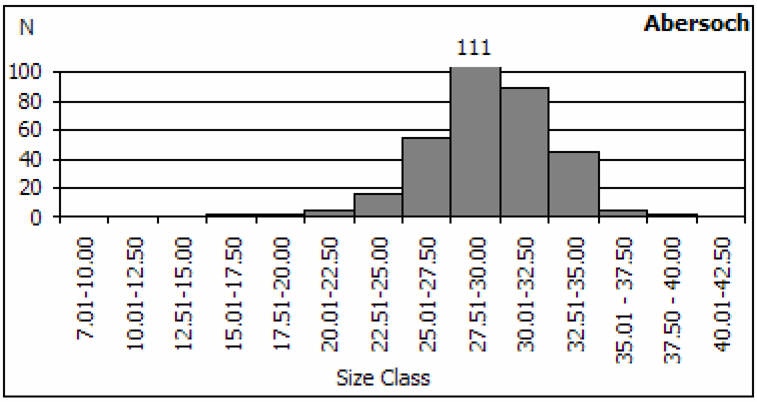
Figure 40 – Photograph of the sampling site (left) and its location (right). Boulders like the one in the picture were the best place for dogwhelks to be found.

Aberdaron is situated on the south west tip of the Lleyn Peninsula (Figure 40). This holiday resort is regarded as being of national importance for its ornithological, botanical and geological interest (Wagimam, 1995). In 2004, the survey was performed on the western part of the bay which is a Precambrian rock outcrop (Saurel, 2002). This year, because of the bad weather, animals could not be collected but in 2004, the study area supported high algae diversity and great numbers of barnacles and dogwhelks. Individual *Nucella lapillus* was found beneath and on boulders vertical surfaces. Both adults and immature dogwhelks were easily found at this sampling site. There was no evidence of pollution or TBT contaminations from small boats, or others, at this study area.

Abersoch

Figure 41 – Photograph of the sampling site (left) and its location (right). Rocky shore where sampling took place: next to the slipway of the old and now disused life boat station. This building has now been converted into a house.

Abersoch is situated on the south east of the Lleyn Peninsula. This village is located on a bay where boat activity is very common since boat excursions to St Tudwal´s Island began. This bay also has moorings for many pleasure craft and some fishing boats (Wagiman, 1995). The study site was located about 1km south from Abersoch, at a place called Porth Bach which is considered to be an exposed shore (Figure 41). There were large numbers of specimens where both adults and immature animals could be easily found on the open rocks surfaces, crevices and under mussels that were extremely abundant. Newly laid egg capsules were also very abundant during the sampling.



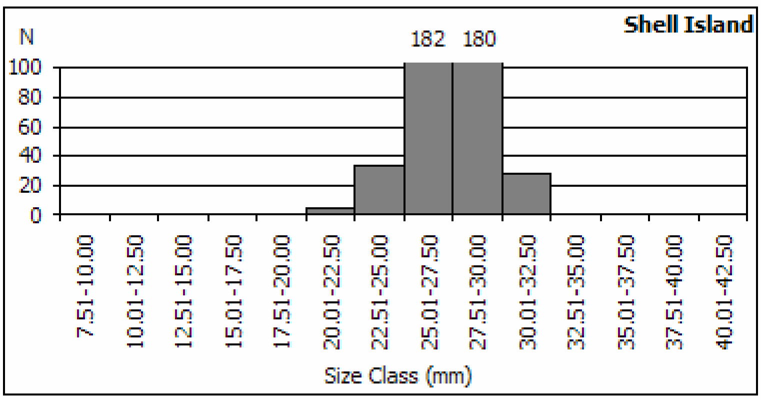
Total number of animals collected during the 3 timed searches: 333.

Histogram 19 – Abersoch. Total number of dogwhelks caught in the three timed searches and number of animals for size class.

Shell Island

Figure 42 - Photograph of the sampling site (left) and its location (right). Dogwhelks were collected under the fucoid algae and mostly on the boulders surface.

Shell Island is located beyond the village of Harlech (~40miles from Caernarfon). The survey site was situated on the rocky shore, which consisted of small and large boulders exposed to wave action (Figure 42). Today, boating at Shell Island is reduced to leisure boats but, in the past, it used to be an important route for big ships. So, this was an important site which had not been assessed before 2004. Individual *N. lapillus* was found beneath and on the vertical surfaces of boulders. This station gives a very good example of communities established beneath boulders Adults and juveniles were easily found within a barnacle and fucoid algal matrix. Algal cover was dominated by fucoid algae and kelps.



Total number of animals collected during the 3 timed searches: 432.

Histogram 20 – Shell Island. Total number of dogwhelks caught in the three timed searches and number of animals for size class.